Pharmacological induction of IL-10 regulatory cells in allergy and asthma

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Pharmacological induction of IL-10 regulatory cells in allergy and asthma

by

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I, Kimuli Ryanna, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Kimuli Ryanna

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Abstract

Allergic and asthmatic disease is highly prevalent in the UK, however current treatments such as steroids, although effective in many individuals, only relieve disease symptoms, and do not provide long-lasting relief. Allergen immunotherapy can provide long-term alleviation of disease symptoms however is only effective in a proportion of patients, carries significant risk of adverse side effects and needs to be given over a prolonged period of time, often several years, for maximal efficacy.

Allergic disease is associated not only with a type 2 adaptive immune response, but also impairment of regulatory T cell function. Allergen immunotherapy is associated with skewing of allergen-specific Th2 responses towards an IL-10 phenotype, suggesting that plasticity of this T cell lineage occurs *in vivo*. Work from our lab has shown that steroids such as dexamethasone, although non-specific in action, augment IL-10 synthesis by CD4+ T cells, a response that is enhanced by vitamin D. The major focus of this thesis was to assess whether established human CD4+ Th2 cell lines could be deviated towards an IL-10+ profile by dexamethasone and 1α25-dihydroxyvitamin D3, and whether these agents might therefore represent appropriate adjuvants to boost the antigen-specific effects of immunotherapy. Combined drug treatment of established Th2 cell lines increased Foxp3+ expression although these cells were not inhibitory in an *in vitro* assay of suppression. In contrast Th2 cells co-cultured with dexamethasone, 1α25-dihydroxyvitamin D3 and IL-10 over a 2-week period deviated towards an IL-10+ phenotype as assessed by qPCR and flow cytometry. Analysis of TCR-Vβ receptor usage suggested this did not represent clonal outgrowth. These cells exhibited strong suppression of Th2 cells *in vitro*, although this was unexpectedly not reversed by addition of neutralizing antibodies to IL-10 or TGFβ to the cell culture.

Analysis of several genes previously identified to be upregulated in freshly isolated CD4+ T cells cultured with dexamethasone and 1a25-dihydroxyvitamin D3 did not reveal comparable expression by Th2-deviated IL-10+ T cells. A transcriptional gene expression array was therefore performed in order to search for biomarkers of these deviated cells and clues as to suppressive mechanisms by which they inhibit Th2 cells proliferation. Genes identified to be of interest included PDCD1LG2, BTLA and several granzymes. Granzyme expression was subsequently validated by qPCR.

Severe asthma is associated not only with Th2, but also Th17 cells, therefore the capacity of 1α25-dihydroxyvitamin D3 and dexamethasone to deviate Th17-associated cytokine production was also assessed. Dexamethasone failed, and indeed could enhance IL-17A synthesis in cultures of CD4+ T cells, which may contribute to severe steroid refractory asthma. 1α25-dihydroxyvitamin D3 inhibited IL-17A synthesis in a glucocorticoid-independent manner.

This work demonstrates that calcitriol and dexamethasone can be used *in vitro* to manipulate T cell plasticity to skew effector phenotypes towards more regulatory phenotypes. This has the potential to tailor and further develop therapeutic allergen specific regimens *in vivo*.

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$1\alpha,25(OH)_2D3$	1 alpha,25-dihydroxyvitamin D3
AhR	Aryl hydrocarbon receptor
AP-1	Activator protein 1
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-CY7 conjugate
APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
BCL-6	B-cell lymphoma 6 protein
c- Jun	Jun gene
c-maf	musculoaponeurotic fibrosarcoma oncogene homolog
CBA	Cytometric bead array
CCR4	C-C Motif Chemokine Receptor 4
CD	Cluster of differentiation
cDNA	complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CO2	carbon dioxide
COPD	Chronic obstructive pulmonary disease
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2 cells
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1
CYP27B1	Cytochrome p450 27B1
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EAR	Early allergic response
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Eosinophil peroxidase
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FCeRI	Fc epsilon RI
FCS	Fetal calf serum
FeNO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in one second
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
GATA3	GATA Binding Protein 3
GITR	Glucocorticoid-induced TNFR family related gene
GMCSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticorticoid receptor

HBSS	Hanks' Balanced Salt Solution
HDAC	Histone deacetylase
IFN(g)	Interferon
Ig(E)	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF4.	Interferon regulatory factor 4
LAR	Late allergic response
LREC	Local research ethics committee
LTC4	Leukotriene C4
MCP	Monocyte chemotactic protein
ml	Millilitre
mM	Millimol
mRNA	messengerRNA
Ng	Nanogram
nmol	Nanomol
PBMC	peripheral blood mononuclear cell
PE	Phycoerythrin
Pg	Pictogram
PGD2	Prostaglandin D2
PMA	Phorbol-12-myristate-13-acetate
PPD	Purified protein derivative
PZLF	Promyelocytic leukaemia zing finger protein
RelA	RelA gene (for transcription factor p65)
RNA	ribonucleic acid
RORC	RAR Related Orphan Receptor C
RORyt	RAR-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription polymerase chain reaction
SPF	Sun Protection Factor
STAT(4)	Signal transducer and activator of transcription 4
TCR	T cell receptor
TGFb	Transforming growth factor beta
Th(1)	T helper cell
TNF	Tumor necrosis factor
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin
ul	Microlitre
UV	Ultraviolet
VD	Vitamin D
VDR	Vitamin D receptor
Vit	Vitamin D

Chapter 1

Introduction

1.1 T helper cells – phenotypic classification

The adaptive immune system, coordinated by T helper cells, has developed to counteract the effects of numerous antigens and microorganisms. These cells provide specificity and memory within the immune system, and direct the function of cells within both the innate and adaptive immune compartments. The functional consequences of T cell activation are largely thought to be beneficial directing the host immune response to combat a large array of pathogens. However, it can also drive disease pathology when aberrant immune responses develop to self-antigens or environmental antigens that do not pose a threat to the host. Conversely, failure to mount adequate adaptive immune responses can also lead to disease pathology, such as in cancer and chronic infection.

The traditional classification of CD4 T helper cell phenotypes, described by Mosmann and Coffman, centred on two classes of T helper cells, derived from naïve T cells through responses coordinated by antigen presenting cells. These cells - Th1 and Th2 cells [1] – were thought to exist in a state of terminal differentiation. In all cases the nature of the T cell phenotype that develops is strongly influenced by the antigen presenting cell and cytokine and mediators within the local milieu[2].

Th1 cell development is classically promoted by secretion of IL-12 from APCs. The IL-12 receptor stimulus leads to the increased expression of STAT4, leading to the increased expression of Th1 associated genes such as IFNγ. TCR stimulus in this setting will lead to increased expression of the transcription factor Tbet (Figure 1.1). A prominent consequence of IFNγ production is to stimulate macrophages and direct them to intracellular killing of pathogens such as viruses and mycobacteria.

Th2 cell development is fostered by the cytokine IL-4, with increased expression of the master transcription factor GATA3, and an additional contribution from cmaf and also IRF4. STAT6 will be the main signalling factor

involved in the immune response, leading to production of the cytokines IL-4, IL-5 and IL-13[3]. Classically the Th2 response has been directed towards action against helminths but may be misdirected against allergens (see later text). Th2 cells also contribute to B cell help within the immune response.

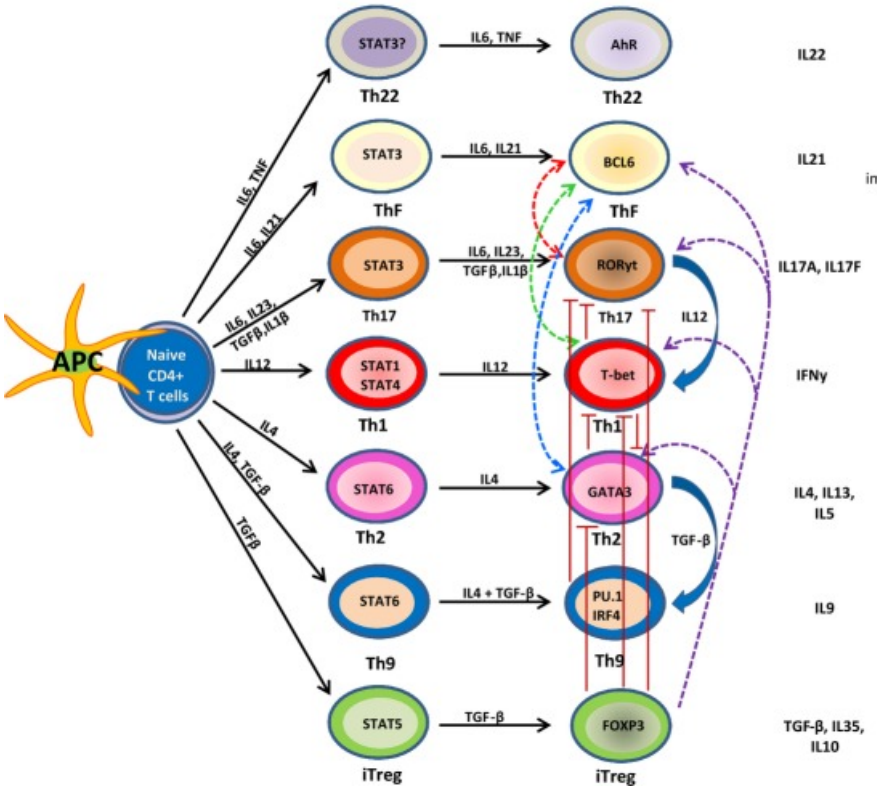
More recently other classes of T helper cell have been identified that are involved in effector responses – these include Th17 cells, Th22 cells, Th9 cells and T follicular cells (Figure 1.1).

Cells have been identified that predominantly secrete IL-17 and IL-22, Th17 cells, with transcription factor RORyt (RORC in humans)[4]. These cells may contribute to the pathological response once attributed to Th1 cells and mediate diseases such as colitis, arthritis and multiple sclerosis[5]. In the mouse, Th17 differentiation is mainly stimulated by TGF beta and IL-6 [6,7]. These cells may be downregulated by Th1 and Th2 cytokines[8] Pathogenic phenotypes in humans are driven by the production of IL1β and IL-23[9] .Th17 and Th22 cells are predominantly directed towards bacteria and fungal pathogens at mucosal sites such as the airways[10,11].

Follicular T helper cells have recently been described. These cells have a specific predilection for lymphoid follicles and they are required for germinal centre development and function, facilitating B cell affinity maturation[12]. They do not have a canonical cytokine to promote development, unlike Th1 and Th2 cells that are stimulated by IL-12 and IL-4 respectively. Early differentiation is promoted by IL-6 (IL-21 in humans), IL-2 and ICOS and depends on TCR signal strength, and development appears to be driven by the master transcription factor BCL-6[13].

The production of Th9 cells is another recently recognised phenomenon, Th2 cell development in the presence of the cytokines IL-4 and TGFβ may result in the production of polarised Th9 cells that secrete IL-9[14].

Figure 1.1 T cell subsets (reproduced from [15])



Most recently, a new phenotype of cells has been identified that secrete the cytokines assigned to the T helper cytokine subsets, but do not have cell surface markers associated with other immune lineages. These cells do not express a T cell receptor. These cells are called ILC cells or innate lymphoid cells. They are found in the blood, lymphoid and lung tissue in the mouse and they have been identified in BAL fluid and lung parenchyma in humans [16,17]. ILCs arise from common lymphoid progenitors in the bone marrow, and require the transcriptional inhibitor Id2 for their development. The cells develop into an ILC precursor that is dependent on the transcription factor PZLF. The cells will develop further into more defined lineages – ILC1 (type 1), that secretes IFNγ, ILC2 (type 2) that secretes IL-6, IL-13 and IL-5, and ILC3 (type 17) that secrete IL-22 and IL-17. Broadly the cells function against similar pathogens noted for the classical T helper subsets (bacteria/viruses for type I, helminths/worms for type 2, and fungi/bacteria for type 17), and by implication where the cytokine production of these cells is aberrant, they may also be involved in a similar spectrum of immune-mediated diseases. Indeed

a growing area of interest is whether ILC2 play a role in the pathology of asthma and other allergic diseases[18-20].

Intriguingly, the discovery of a larger number of subsets of CD4 effector T cells than were first described may reflect increasing plasticity of T cell phenotypes rather than final differentiation [21,22].

1.2 Regulatory T cells

Recent developments in immunology suggest that CD4+ T cells can differentiate into a broader repertoire of phenotypes, which include cells with a suppressive phenotype as well as effector phenotypes, with cytokine profiles that differ from the previously established dual model of Th1 versus Th2 effector cell phenotypes. They can be broadly categorised into natural Treg and peripheral, or induced Treg[23]. The ontogeny of regulatory T cells, particularly those derived from the periphery, is still uncertain, but they may derive from naïve T cells for which thymically derived natural CD4+Foxp3+ Treg provides the obvious example, and may also derive from naïve or previously activated or even lineage committed cells in the periphery.

1.2.1 Natural Treg

Sakaguchi identified a subset of T cells originating from the thymus that suppressed the immune response. Knockout (athymic) mice that did not express this T cell subset developed catastrophic autoimmunity leading to early mortality. The suppressor, or regulatory T cells had high constitutive expression of the CD25 marker and this was noted to be an early marker to define this cell population[24]. The Foxp3 transcription factor was later identified in scurfy mice further demarcating regulatory cells as a distinct population with

evidence that knock out of Foxp3 also leads to autoimmunity[25]. Further evidence was provided by the discovery of a severe autoimmune disease in humans linked to a mutation in the human Foxp3 gene with a subsequent decrease in the regulatory T cell population, leading to an X linked syndrome of immune dysregulation, polyendocrinopathy and enteropathy (IPEX) [26].

Thymic Treg cells appear to be a separate lineage from naive T cells that go on to become effector T cells upon stimulation. One theory is that a subpopulation of cells that have high affinity T cell receptors for self antigen express Foxp3 following contact with medullary stromal cells and become Treg[27]. The function of the thymic medullary cells and other thymic accessory cells is proposed to allow promiscuous expression of a large array of (self) antigens enabling the development of Fox3 Treg with the potential to control inappropriate immune reactions[28]. This process may require the interaction between CD154 and CD40[29]. Other investigators have found that Foxp3 thymocytes can develop in the cortex even if medullary migration has been blocked[30].

Maintenance of the Treg pool requires IL-2, for persistence of the phenotype[31] as well as suppressive function through competition for cytokines[32]. Production of new Treg cells may occur in the thymus but thymic involution secondary to aging will reduce this number to a very small fraction in adult humans. It is therefore likely that the new nTreg will be sourced from induced cells in the periphery.

One problem with phenotypic characterisation has been the specificity of CD25 high and Foxp3 as markers for the natural T reg population. These markers can be expressed in activated T cell populations[33] and therefore further attempts have been made to characterise this population in more detail. Markers that have been postulated include CTLA 4, GITR and CD62L[23], but a commonly used combination that does not require cellular permeabilization (which Foxp3 does) is CD4+CD25high, CD127low/negative[34].

Expansion of the natural T regulatory cell population is dependent on appropriate stimuli from dendritic cells[35] and maintained by TGF- β [36].

1.2.2 Peripheral Treg

The regulatory T cell repertoire has been further categorised to reflect the presence of regulatory T cells that differentiate in peripheral tissues rather than the thymus. This may reflect a need to combat harmful immune responses that occur as a result of exposure of sites to antigen, with limitation of immunopathology[37], with natural Treg remaining *in situ* to combat autoimmunity. Both Foxp3+ve[38] and Foxp3–ve[39] populations have been described. Although the cells do not originate in the thymus, they may be recent thymic emigrants[40]. It has been shown that effector T cells in skin stimulated through the injection of PPD can be anergised through further inoculation with PPD and converted towards a suppressive phenotype indicating that Treg can be derived from locally inflamed tissue. IL-10 Treg have been described in the periphery, and have been variously proposed to be stimulated by IL-10[41] , exogenous drugs[42] and through stimulation of the receptor CD46 [43] (reviewed in [44]).

1.2.3 Mode of action of regulatory T cells

Regulatory T cells (Treg) can act in a number of ways to suppress effector cell function. They can secrete inhibitory cytokines, such as IL-10 or they can effect metabolic disruption through cytokine deprivation, e.g. IL-2. They may act directly via cytolysis (e.g. via granzymes) or they may act indirectly through targeting dendritic cells (Figure 1.2).

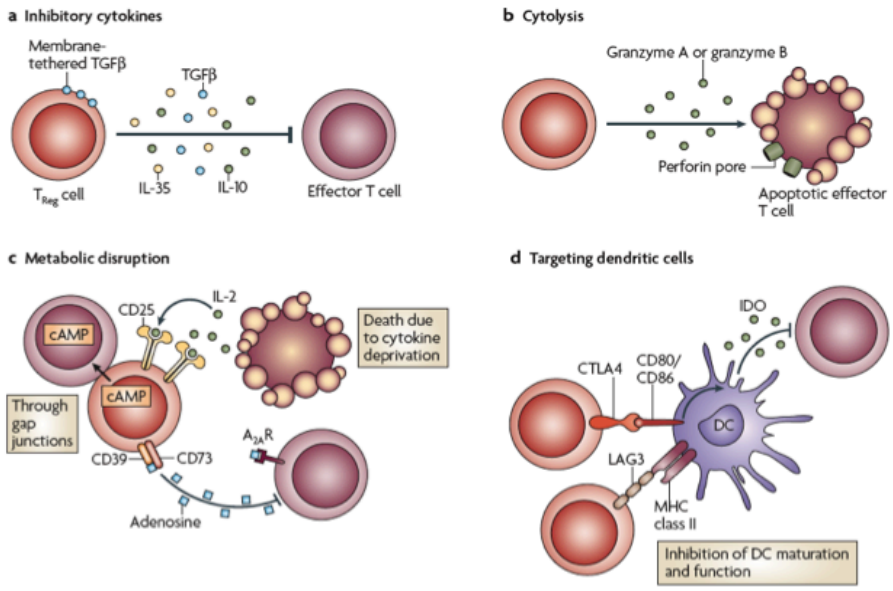


Figure 1.2 Mode of action of Tregs (reproduced from[23]).

1.2.4 Stability of regulatory T cells

Treg do not proliferate as strongly as effector T cells *in vitro* although the exact duration of the Treg life cycle is unknown. Persistence of the Treg phenotype involves epigenetic modification of Foxp3 – the numbers of these cells in the thymus are increased by the use of a histone deacetylase inhibitor, and Treg function is enhanced by increased acetylation achieved through the nuclear export of histone deacetylase 9 (a class two histone deacetylase) which normally binds to foxp3 in resting T cells[45]. Methylation is another modification that may be significant as it has been shown that natural Tregs exhibit demethylation, whereas activated T cells and peripheral TGFβ induced Treg do not [46 47].

Most experiments *in vitro* have derived Treg from naive T cells as described above with the addition of specific cytokines such as TGFβ, but the population of human RA+ cells *in vivo* from which the Treg will be derived will be small[48]. nTreg have very short telomeres in comparison to the general CD4+ population or memory cell population and therefore it is unlikely that this population is generated from a population that replicate indefinitely. This raises the possibility that Treg may be generated from proliferating memory cells[49].

There is evidence of interaction between effector T cell differentiation and regulators of Treg. Mantel[50] demonstrated that TGF-β mediated Foxp3 induction is inhibited by overexpression of GATA-3 in a murine model, a finding replicated by Wei[51] .Wei also found that the Foxp3 induction also downregulates the Th1 master regulator T-bet. Reciprocal regulation of Foxp3 and RORγt/RORC expression has also been identified[52]. Expression of Foxp3 may have an inhibitory effect on IL-10 expression in asthma[38] which may indicate different stages of Treg activity during the development of aberrant immune responses. The literature indicates that this phenomenon may have biological significance in that the IL-10 may be important for

immunoregulation at environmental interfaces, whereas Foxp3 is more important for systemic autoimmunity[53].

1.3 Asthma and allergy

1.3.1 Epidemiology of asthma and allergy

Allergic disease encompasses a broad range of organ pathology including conjunctivitis, rhinitis, atopic dermatitis, food allergy, anaphylaxis and asthma. The prevalence of allergic diseases has reached epidemic proportions worldwide[54,55]. The prevalence of asthma alone is 235 million worldwide[56]. Although allergic diseases such as hay fever were noted as far back as 1870[55] , the incidence was low. The mid-20th century onwards marked the beginning of a sharp escalation in the prevalence and incidence of these diseases[57], predominantly in Westernised countries. This has led to significant morbidity and mortality [58].

The notable increase in allergic diseases over the last 50 years has stimulated interest in factors that may have contributed towards modulation of immune responses. One major hypothesis that has been postulated is that decreased exposure to allergens and antigens in early life may contribute towards the development of an allergic response in infancy and beyond. A recent paper[59] has shown that the introduction of allergen during infancy, specifically peanut extract, leads to a decrease in allergic responses to peanut at 60 months. Strachan, in his keynote paper[60], recognised that there was an association between the number of older children in the household and the prevalence of hay fever in the first year of life. The increase in atopy persists even in later life[61]. There appears to be an allergic march with age, which begins with atopic dermatitis and food allergy, culminating in asthma and allergic rhinitis in later life[62]. A lack of exposure to microbes in early life may

skew responses away from Th1 towards Th2 responses, at least in affluent countries[63]. In low income countries, where they may be increased exposure to parasitic infection, there may be an increase in IgG4 responses, with hyporesponsiveness towards antigens[64]. However, a concept that is increasingly being considered is that infectious history may influence the frequency of regulatory T cells, based in part on the evidence that not only allergic, but also a range of autoimmune, conditions have increased in prevalence during a similar time period[65].

1.3.2. The Th2 paradigm and evolution into broader phenotypes

The phenotype of allergy is signified by inflammation directed towards a harmless allergen. The allergic response is classically divided into two phases (figure 1.3) - the early, or acute allergic response (EAR), and the late allergic response (LAR). Allergen-specific IgE is bound to the high affinity IgE receptor (FCεRI) on mast cells and basophils. Cross linking of IgE by allergen leads to mast cell degranulation with resulting discharge of mediators including histamine and cysteinyl leukotrienes with instantaneous effects (e.g. bronchoconstriction)[66]. This early phase in asthma is orchestrated by the initial secretion of tissue specific cytokines from the airway, namely IL-25, IL-33 and TSLP[67-70]. Classically, the late phase is denoted by an influx of Th2 cells with release of cytokines IL-4, IL-5, IL-9 and IL-13, and also activation and degranulation of eosinophils leading to a more sustained response and damage of surrounding tissues.

Allergic rhinitis, conjunctivitis and dermatitis broadly fit into the classical Th2 paradigm in addition to the majority of people with mild allergic asthma[71]. In recent years researchers have noted that the characterization of asthma is wider with many more phenotypes of asthma leading to disease, which account for the spectrum of severity of the disease and lack of responses to classical glucocorticoid treatment[72]. The patients with the most severe

disease are those who are at high risk of recurrent exacerbations and hospitalisation[58].

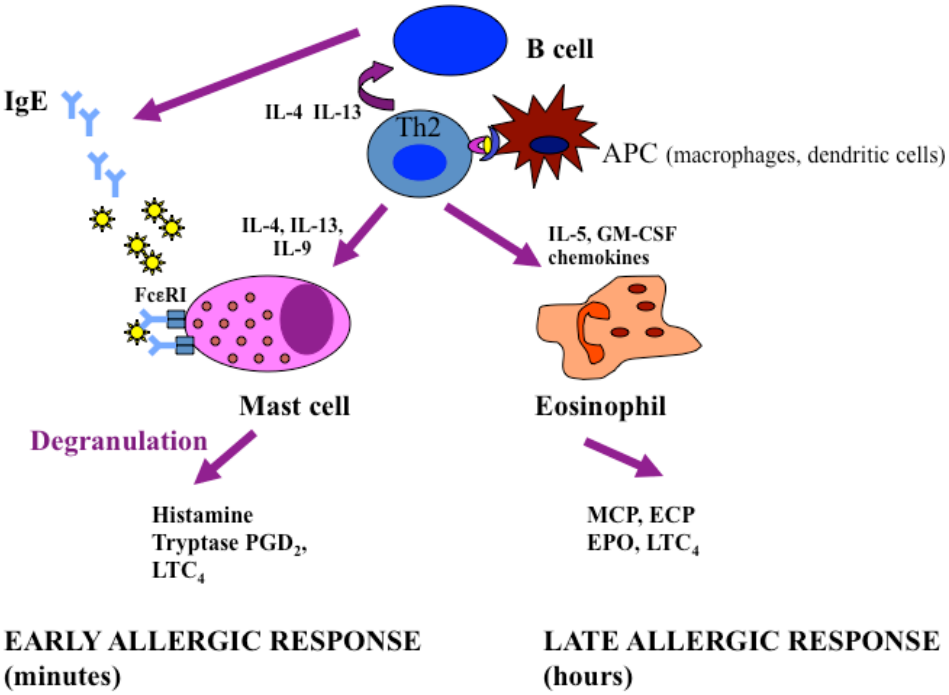


Figure 1.3 Early and late allergic response

Some patients with severe allergic asthma may have a phenotype that signifies high IgE production. Other patients will also have a Th2 high response, with significant eosinophilia contributing to their disease phenotype[73]. Th2 low phenotypes in asthma might reflect a switch from Th2 pathways to other effector pathways with a different cellular response including Th17 cells, that stimulate antibacterial defences within the mucosa, and exhibit a mixed cellular response in the airways with the presence of both neutrophil and eosinophil effector response[74].

1.3.3 Classical treatments for asthma and allergy – glucocorticoids

The mainstay of treatment for allergic diseases including asthma is glucocorticoid therapy. Clinical researchers in the early to mid-20th century

demonstrated that cortisone had a beneficial effect on asthma[75] and other allergic diseases[76] and over time a broader range of glucocorticoids were developed, in both oral and inhaled forms, that could be administered for treatment of these conditions[77].

1.3.4 Mechanism of action of glucocorticoids

Glucocorticoids are steroid hormones that diffuse through the cell plasma membrane and mediate their anti-inflammatory actions via the glucocorticoid receptor[78]. Several variants of the glucocorticoid receptor exist as a consequence of alternative splicing. Two main isoforms of glucocorticoid receptor have been characterised, GRα and GRβ. GRα is widely distributed. In T cells, it is located within the cytoplasm and ligand binding induces translocation to the nucleus. . Glucocorticoid receptor complexes formed of homodimers of GRα then bind to glucocorticoid receptor elements on genes within the target cells, leading to repression of inflammatory pathways, and stimulation of others. This broad range of activity enhances the efficacy of its anti-inflammatory profile. Negative response elements have been described but only a few have been thoroughly characterised. GRβ, on the other hand, is predominately nuclear and, in general, is expressed at a lower level than GRα within the cell. GRβ is able to bind to DNA but appears to be unresponsive to glucocorticoids and the upregulation of this isoform will act as a competitive inhibitor of glucocorticoid action[78].

The activated glucocorticoid receptor can also prevent the expression of pro-inflammatory genes by binding to the RelA and c- Jun subunits of NF-κB and AP-1, respectively[79]. Glucocorticoids are thought to participate in chromatin remodelling. Ligand-bound glucocorticoid receptors can recruit histone deacetylases, for example HDAC2, leading to a reduction in pro-inflammatory gene transcription through the deacetylation of histones[80]. The actions of the glucocorticoid receptor will result in down regulation of transcription factors for the effector T cell cytokines, such as IFNγ, IL-4, IL-5, IL-13[79].

1.3.5 Glucocorticoid resistance

A proportion of asthmatic patients fail to respond to therapy with glucocorticoids, termed steroid resistant individuals. Equally, steroid insensitive patients exist, who require high dose, prolonged treatment with glucocorticoids to establish clinical efficacy. Approximately 5-10% of patients have asthma that is refractory to steroid treatment[16]. Although this disease state is rare, these subjects have major morbidity and mortality[81].

Several theories have been proposed to explain the phenomenon of steroid insensitivity. IL-2, IL-4 and IL-13 have all been linked to steroid resistance as the expression of these cytokines is elevated in bronchial biopsies of steroid resistant asthmatic subjects. IL-2 and IL-4 have been implicated further by *in vitro* studies. For example, this combination of cytokines, acting via p38 MAP kinase, phosphorylates glucocorticoid receptors, causing a decrease in glucocorticoid binding affinity within the nucleus. Another explanation is that the dominant-negative GR isoform GRβ contributes to steroid insensitivity. Elevated expression of GRβ has been reported in PBMCs and BAL fluid from glucocorticoid resistant, compared with glucocorticoid sensitive asthmatic patients[82].

Other risk factors for glucocorticoid insensitivity include cigarette smoking, genetic predisposition and allergen exposure. It is probable that multiple parameters influence the balance between glucocorticoid sensitivity/resistance in asthmatic individuals. Suitable alternative therapies are therefore necessary for this important group of patients.

A number of biological strategies have been developed to identify antibodies that block cytokines or receptors involved in the Th2 pathway, as well as IgE mediated disease, and more recently TNFα and IL-17. A table of antibodies that have been trialled follows below:

Table 1 Biological treatments for asthma

Target	Biological therapy/antibody	Outcome	Ref
IL-4 and IL-13	IL-4Rα – dupilumab	Decreased exacerbations of asthma and FeNO	Wenzel S et al, 2013[83]
IL-13	IL-13 – lebrikizumab	Increased FEV1	Corren J et al, 2011[84]
IL-4	Mutant IL-4 – pitankira	Decreased asthmatic response	Wenzel S et al, 2007[85]
IL-13	IL-13 – Tralokinumab	Increased FEV1	Piper E et al, 2013[86]
IL-5	Anti-IL-5 mepolizumab	Decreased exacerbations in patients with high sputum eosinophils	Ortega H et al, 2014[73]
IL-5	Anti IL-5 reslizumab	Decreased exacerbations in patients in moderate to severe eosinophilic asthma	Castro M et al, 2015[87]
IL-5	Anti IL-5Rα – benralizumab	Decreased eosinophils in sputum, bone marrow, blood	Laviolette M et al, 2013[88]
IgE	Anti-IgE mAb - omalizumab	Decreased asthma exacerbations	Humbert M et al, 2005[89]
Additional strategies in asthma			
IL-9	Anti IL-9 enokizumab	Discontinued – no effect	Boyman, O (review)[90]

IL-17	Anti IL-17R - brodalumab	Moderate to severe asthma – no effect	Busse WW et al, 2013[91]
TNF alpha	Anti TNF alpha; golimumab	Unfavourable risk: benefit profile; Discontinued due to adverse events, including one death and malignancies	Wenzel S et al, 2009[92]
TSLP	Anti TSLP – AMG 157	Reduced FeNO, blood and sputum eosinophils	Gavreau et al, 2014[93]

Successful glucocorticoid therapy is associated with an increase in production of the immunomodulatory cytokine IL-10. IL-10 suppresses inflammation through reduction of Th2 cytokines and also effects on macrophages and dendritic cells[44]. Steroid resistant patients exhibit a decreased capacity to generate IL-10 and strategies to overcome this deficit are advantageous[94] Administration of IL-10 itself is impractical due to its short half-life[95], and therefore strategies to increase T cell production of IL-10 could prove more beneficial. This can be done either through the use of other pharmacological agents to augment the IL-10 response, or to increase the number of IL-10 Treg or Tr1 in the population of T helper cells.

1.3.6. Allergic and asthmatic disease as a sequelae of impaired regulation – deficiency or impaired activity of regulatory T cells

Studies have shown that allergic disease states can be associated with a decrease in the number and function in the number of nTreg. Ling et al demonstrated that patients with hay fever had reduced levels of $CD4^+CD25^+$ cells with less suppressive capacity during the hay fever season[96] a finding also demonstrated by Grindebacke in relation to birch pollen[97]. Hartl showed that the $CD4^+CD25^+Foxp3^+$ Treg in children with asthma were reduced in the

airways of these children, and had decreased suppressor activity[98]. Restoration of immune function can be seen with adoptive transfer of natural Treg in mouse models of allergic disease[99], and small clinical trials are being established to determine the safety of Treg transfer in humans, although these trials are being performed within the field of transplantation medicine.

1.3.7 Allergen immunotherapy

In contrast to glucocorticoid therapy, allergen immunotherapy is a more specific form of therapy directed at treatment of allergic disease, which may have lasting effects after treatment cessation. It is most efficacious in patients who have allergic disease mediated by IgE, such as rhinitis, venom hypersensitivity and conjunctivitis. Research has indicated that it can be effective in asthma but this is not without risk of significant side effects, including fatality[100] and therefore methods to enhance the safety and efficacy of this therapy would be of great benefit to patients.

The therapy is administered via subcutaneous injections, or more recently the sublingual route[100] . Regimens involve a prolonged period of administration in order to achieve maximal efficacy and immunological desensitisation. Therapies exist for the common allergens including grass pollen and bee venom, as well as house dust mite[101]. It is most efficacious if therapy is directed against one allergen rather than multiple allergens[102].

Activated T cells play an important role in the immunological mechanisms that drive allergen tolerance in the context of allergen immunotherapy. Initial theories suggested that successful immunotherapy was stimulated by a decrease in IL-4 and IL-5 production by Th2 cells and also a shift toward Th1 cell production with increased IFNγ[103]. More importantly in the context of this work, allergen immunotherapy stimulates production of IL-10, which is predominantly effected through the increased production of Tr1 cells. These cells also produce TGFβ[103-106]. Immunotherapy also reduces the number of mast cells in tissues and their mediator release, as well as decreasing the

production of allergen specific IgE from B cells, and increasing the frequency of blocking antibodies IgG1, IgG4 and IgA.

1.4 Vitamin D

Vitamin D is a secosteroid hormone which plays an important role in bone and calcium metabolism[107] but it is increasingly recognised that the vitamin has immunomodulatory effects that impact on many different systems within the body.

Vitamin D can be obtained through two sources: exogenous foodstuffs in the diet (e.g. oily fish) with a low yield, but predominantly via conversion of 7 deoxycholesterol in the skin on reaction with UV light. Vitamin D is converted to 25 hydroxyvitamin D in the liver and the last step in conversion towards 1α,25-dihydroxyvitamin D3, the active form of vitamin D, takes place in the kidney, upon the action of CYP27B1. This enzyme is predominantly present in the kidney, but also present within immune cells such as macrophages and T cells (Figure 1.4).

Vitamin D exerts its effects by binding to the cytosolic vitamin D receptor (a member of the nuclear steroid/thyroid hormone receptor family)[107] and then heterodimerises with receptors of the retinoic X receptor family. The complex then translocates to the nucleus where binds to vitamin D response elements These elements are present in many target genes and binding will influence gene transcription. Binding of the vitamin D complex to response elements may be responsible for control of 3% of the genome in mice and humans[108].

Finally, the active form of vitamin D is metabolised to its inactive form by the enzyme CYP24A1.

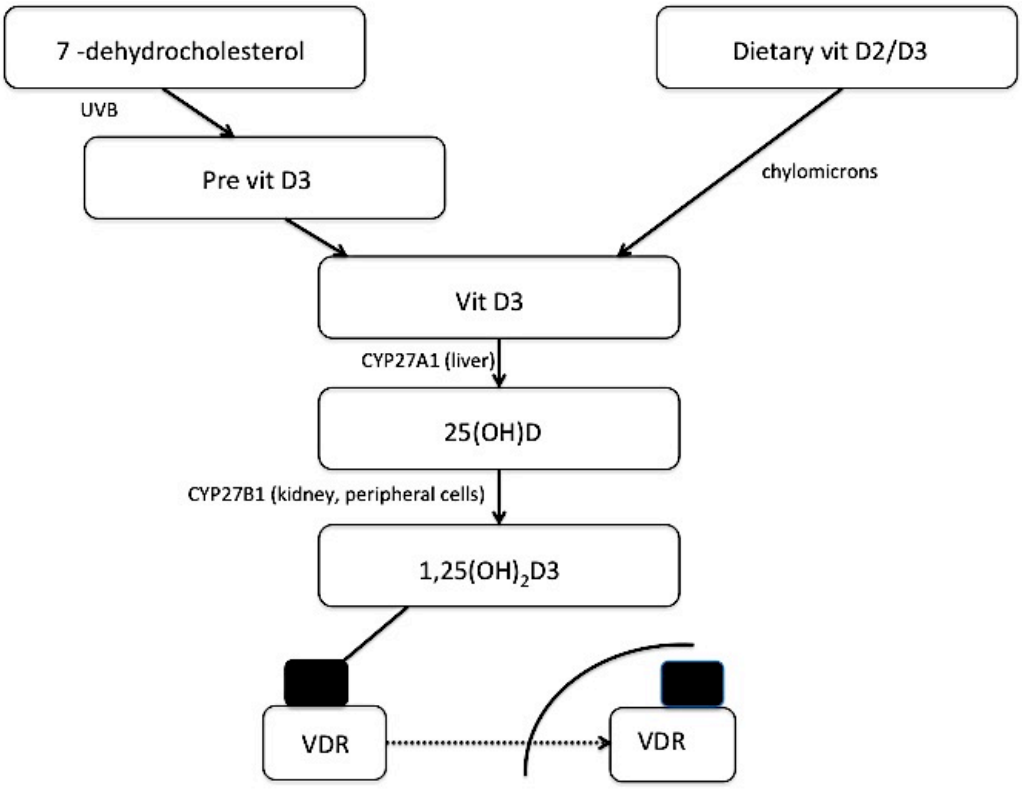


Figure 1.4 Metabolism of vitamin D

Studies have shown that there is a widespread level of vitamin D deficiency[109], particularly in regions that are not exposed to very much sunlight, predominantly in countries at the extremes of northern latitude (very little production of vitamin D in areas beyond a latitude of 35° in winter[110]), although deficiency may be more widespread due to a global trend towards more activity indoors with decreased exposure to sunlight. Countries in regions that do have sufficient sun exposure may be affected by the requirement for sunscreen to be used in those people who are susceptible to skin cancer, which will limit the capacity for UV light to penetrate the skin and begin the first step in the conversion pathway towards vitamin D (SPF greater than 15 leads to greater than 90% absorption of UVB)[111]. In countries with high levels of sunshine, cultural and other factors are likely to influence vitamin D status and these include dark skin pigmentation, excessive clothing, restricted outdoor activity, high levels of atmospheric pollution, and low dietary intake of vitamin D and calcium[107].

There is increasing interest in the role of vitamin D status and disease. The optimal level of vitamin D has yet to be determined[112]. Severe deficiency in children less than 25nmol/l (10ng/ml) will lead to rickets, and in adults will lead to osteomalacia. There is, however, increasing evidence to suggest that higher levels of vitamin D may be required for optimal bone health and to maintain immunological health[113].

Epidemiological studies have linked vitamin D deficiency with autoimmune diseases such as multiple sclerosis[114], in addition to an increased incidence in some cancers and also cardiovascular disease[107]. Vitamin D status is linked to respiratory health, as seen in the Black study[115] and there is data suggesting that vitamin D deficiency may be related to an increase in asthma[116]. There is also circumstantial evidence to suggest that vitamin D deficiency can be associated with severity of chronic obstructive pulmonary disease although this observation may reflect inactivity as a cofounder[117]. COPD and asthma have also been associated with polymorphisms in vitamin D binding protein[118]. Vitamin D may also play a role in defence against infectious disease in respiratory patients[119].

The effect of vitamin D in allergy has been controversial. Some authors have suggested that vitamin D may enhance Th2 responses in culture[120], but contrasting evidence has suggested that VDR knockout mice do not develop Th2 responses[121]. While there are the beneficial effects to respiratory health, as outlined above, this may be a U shaped curve effect. Researchers have noted that IgE levels can be elevated at the extremes of vitamin D metabolism. Elevated levels of IgE have been seen at less than <25 nmol/l as well as very high levels >135 nmol/l. However, more recent data would suggest that restoration of vitamin D levels will help to combat allergic disease, particularly in children[122,123].

1.4.1 Vitamin D and effects on immune function

Vitamin D, through binding to VDR and acting through VDR response elements (VDRE) affects a number of pathways in both the innate and immune systems.

The active form of vitamin D, calcitriol or 1α,25-dihydroxyvitamin D3, is able to act on the innate immune system by downgrading inflammatory responses[124] and augmenting the production of antimicrobial proteins. TLR expression on monocytes is downregulated in the presence of calcitriol[125]. Expression of the antimicrobial peptide cathelicidin is increased and the VDRE is noted to induce expression of this protein in monocytes, keratinocytes and neutrophils[125,126]. The defensins are also increased following treatment by calcitriol[127].

Vitamin D can also modulate T lymphocyte function. Many of these effects are likely to be mediated by the effects of vitamin D on dendritic cells function [128]. Calcitriol inhibits T cell proliferation and IL-2 production. It has effects on Th1 cell cytokines with a decrease in IFNγ production in mice and humans. Calcitriol has also been noted to decrease IL-17 production in animal models of colitis and uveitis. The situation with Th2 cytokines is slightly more complex in that certain studies have shown persistence or enhancement of Th2 cytokines *in vitro* and in animal models. This may be due to the concentrations used as vitamin D is seen to lower Th2 cytokines at levels of 10^{-7} M and below in longer term cultures[39,129]. Overall, experimental data suggesting that vitamin D may enhance Th2 responses is not supported by clinical data. Indeed a negative correlation between vitamin D status and IgE responses, particularly in paediatric cohorts has been reported[122,123]. It is therefore plausible that additional (Th2-specific?) regulatory mechanisms controlled by vitamin D may account for this discrepancy although further research is needed in this area[130].

Work from the Hawrylowicz laboratory that forms the basis of the current study includes reports that the use of vitamin D in combination with dexamethasone leads to restoration of IL-10 production in steroid resistant asthmatic patients[131]. The combination of the active form of vitamin D3, calcitriol, and dexamethasone has been used to deviate naive T cells towards a regulatory T cell phenotype with abrogation of Th1[42] and Th2[131] effector responses. Vitamin D also promotes a range of immune regulatory functions, both innate and adaptive (see Figure 1.5).

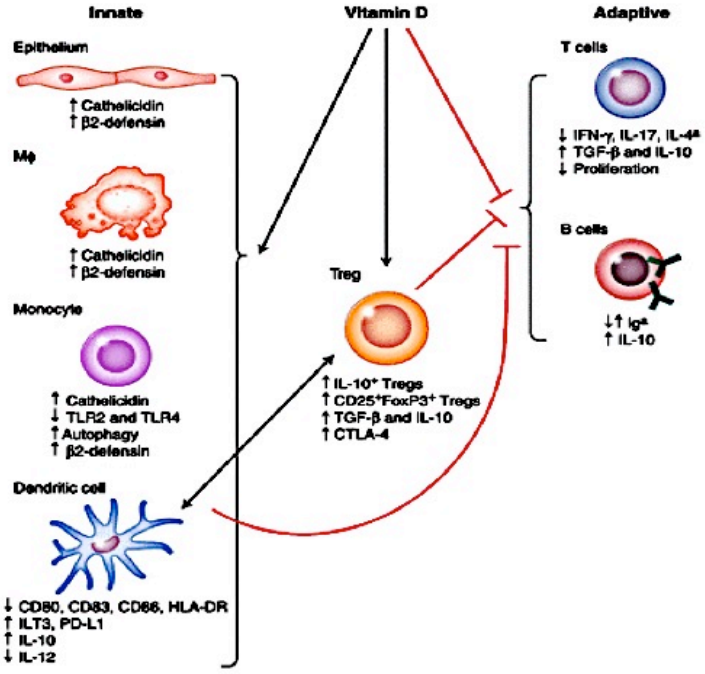


Figure 1.5 Immune actions of vitamin D (adapted from [132])

1.5 Hypothesis and Aims

The treatment of allergic disease is complex. Current treatments that have a broad effect on multiple pathways, such as glucocorticoids, are not allergen specific and they are not without morbidity. The development of novel treatments has focused on agents directed at single targets (e.g. anti IL-5, anti IgE) with variable efficacy in modifying disease. Our increasing knowledge of the immunology that underlies these diseases recognises the multiple interlinked pathways that contribute to the disease process. Allergen specific therapy currently used in the clinic, such as immunotherapy, targets immune responses specific for the disease provoking antigen (allergen), but also carries risk of morbidity and mortality.

Vitamin D, possibly in concert with dexamethasone, has effects on a number of innate and adaptive pathways. Our laboratory has previously described the capacity of these agents to promote a regulatory phenotype in peripheral blood derived CD4+ T cells. The studies in this thesis address the novel question of whether these mediators also have the capacity to deviate more differentiated T effector cell phenotypes implicated in allergic and asthmatic disease, towards a regulatory profile, which would enable these cells to act on a number of effector pathways in tandem, with potential to facilitate this change endogenously in an allergen-specific manner.

Aims

- Investigate whether established effector Th2 cell lines can be deviated in culture towards a regulatory phenotype using vitamin D in the absence or presence of dexamethasone and IL-10.
- Characterise the molecular phenotype of Th2 cells deviated towards a regulatory phenotype in order to determine the mode of action of regulation.
- Determine the capacity of vitamin D to alter effector T cell responses other than the Th2 response, i.e. Th17 responses, in order to explore

the capacity of vitamin D to influence the sensitivity of the response to steroids in other effector T cell systems

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Isolation of peripheral blood mononuclear cells

Ethics approval for this work was obtained from Guy's Hospital Research Ethics Committee (LREC study 1/4/10) and full informed consent was obtained from all donors. Donors were aged 18 or over and were either healthy or atopic. Atopic individuals were defined by the presence of a positive wheal at 15 minutes after skin prick testing to ≥1 of a panel of aeroallergens (cat, grass pollen, silver birch, dog, house dust mite, Alternaria, Cladosporium and Aspergillus; Soluprick, ALK, Denmark) performed with diluent (saline) and histamine controls. Peripheral blood (120 mls) was collected from human donors by venepuncture and anticoagulated with sodium citrate (Sigma-Aldrich Co. Ltd, Poole, UK) at a ratio of 10:1 and diluted with Hank's buffered salt solution (HBSS; Life Technologies, Gaithsburg, MD) in a 1:1 ratio. Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient (Lymphoprep®, Axis Shield, Oslo, Norway) by layering the blood on the Lymphoprep® at a ratio of 3:1 followed by centrifugation at 800g for 20 minutes at 18°C. The cells were recovered from the interphase and washed twice with HBSS with 2% fetal calf serum (FCS; PAA laboratories, Pasching, Austria), first 600g for 10 minutes at 4°C, then 200g for 10 minutes at 4°C.

2.1.2 Isolation of CD4+ T cells by positive selection using magnetic beads

CD4+ T cells were isolated using Dynal CD4 Positive Isolation Kit (Dynal, Oslo, Norway). PBMC were incubated with CD4 coated Dynabeads® at a bead to target ratio of 3:1 (25µl per 10^7 PBMC) for 20 minutes at 4°C under continuous rotation. The sample was then placed on a magnet for 2 minutes. The supernatant containing the CD4+ depleted cells was discarded and the bead-bound cells were washed six times with 2%FCS/HBSS by application to

the magnet. The bead-bound cells were then resuspended in 500µl of 2%FCS/HBSS, 200µl of DETACHaBEAD® (Dynal) was added and the cells were incubated for 45 minutes at room temperature under continuous rotation. The sample was applied to a magnet and the supernatant containing the released, purified CD4+ cells was transferred to a fresh tube. The beads were washed twice in 2%FCS/HBSS to collect residual cells. The CD4+ cells were then washed twice in 2%FCS/HBSS at 200g for 10 minutes at 4°C to remove any of trace of the DETACHaBEAD® solution. Purity was routinely assessed as >98% by flow cytometry analysis (BD FACSCalibur, figure 1).

2.1.3 Isolation of cell populations by cell sorting

Isolation of CD4+CD45RA+ cells

Human peripheral blood mononuclear cells were isolated as described. The cells were counted and then stained with the required combination of sterile antibodies at a ratio of 40µl per 10^8 cells and incubated for 20minutes on ice. Labelled antibodies used for sorting were used as follows: FITC CD45RA (HI100), PE CD4 (RPA-T4), APC CD45RO (UCHL1), APC-Cy7 CD14 (MΦP9) (all antibodies BD Biosciences).

Initial work to adjust compensation involved compensation beads (BD), in accordance with the manufacturer's instructions. In brief 1 drop each of negative and positive (anti Mouse IgG) compensation beads (BD) and mixed with 100ml of 2% HBSS. Each tube was then incubated with 2µl of a single antibody for 20 mins at room temperature. A small aliquot of unstained cells was kept as a negative control.

This method was superseded by the use of cells isolated from the PBMCs prior to staining, i.e. four individual tubes containing one 2µl aliquot of PBMCs were mixed with 100µl of HBSS per tube and stained with a single antibody

as above. This was done to minimise any voltage changes between processing beads and using the cell sample.

Purity was assessed as >98% for CD4+CD45RA+ T cells.

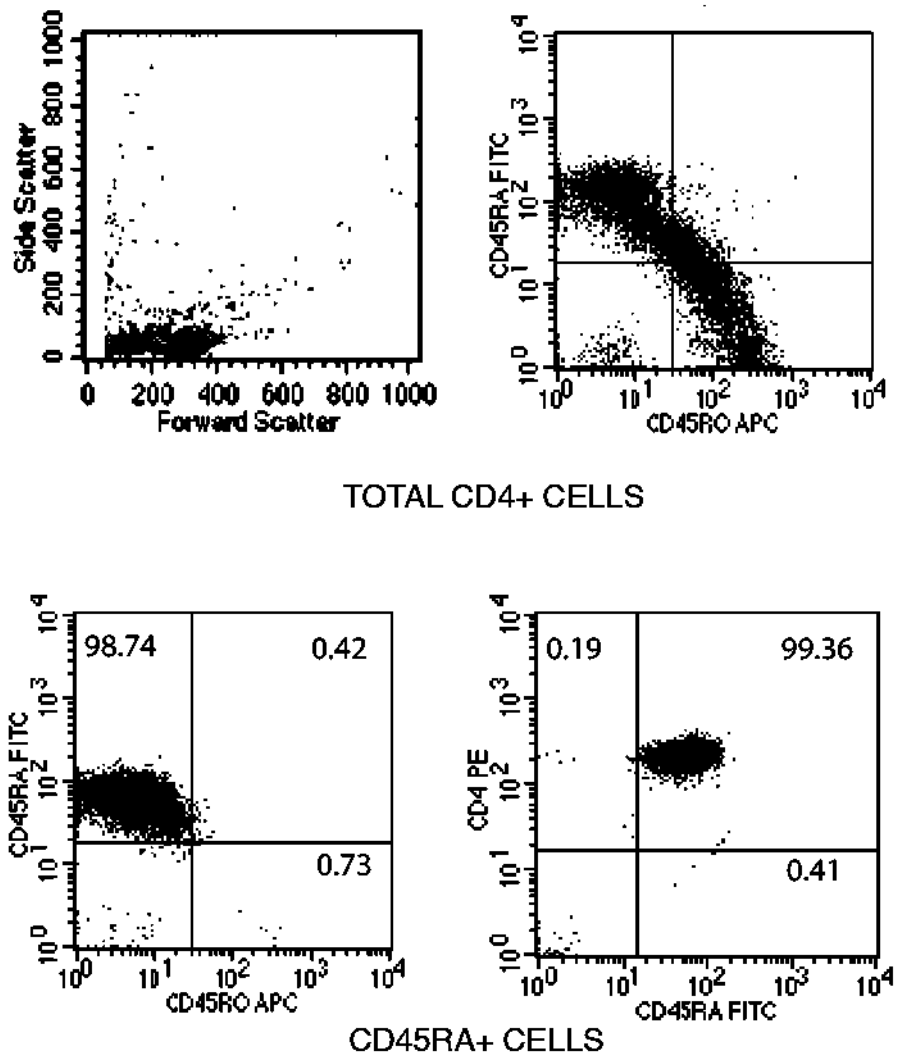


Figure 2.1 Isolation of CD45RA cells by flow cytometry.

CD45RA+ cells were isolated from PBMCs using flow cytometry. Purity of the CD45RA+ cells was 99%. Data shown from one representative experiment of three.

2.1.4 T cell culture conditions

Culture of total CD4+ T cells

Isolated cells were counted using a haemocytometer with dead cells excluded by the use of Trypan Blue staining. Purified cells were re-suspended at a concentration of $1x10^6$cells/ml in RPMI 1640 (Life Technologies), supplemented with 10%FCS, 2mM L-glutamine (Life Technologies) and 50mg/ml gentamycin (Sigma-Aldrich; 10%FCS/RPMI). Cells were stimulated with plate-bound anti-CD3 (1mg/ml; clone OKT3; purified in-house) and rIL-2 (50U/ml; Eurocetus, Harefield, UK). Feeding occurred on day 3; half the volume of culture medium was removed and replaced with 10%FCS/RPMI with 40U/ml rIL-2.

Generation of human Th2 cell lines *in vitro*

Purified CD45RA+ cells ($1x10^6$/ml) were stimulated with plate-bound anti-CD3 (1µg/ml; clone OKT3; made in house), anti-CD28 (2µg/ml; clone 15E8; Sanquin, Amsterdam, Netherland) and rIL-2 (50U/ml; Chiron, Emeryville, CA). rIL-4 (12.5ng/ml; R&D Systems), anti-IFNγ (5µg/ml; clone 7R2/A4; ECACC). without or with 5ng/ml IL-10 (R & D) from day 0. Lines were restimulated every 7 days.

Cultures were restimulated weekly without (Th2) or with addition of 1α, 25-dihydroxyvitamin D3 (Biomol) plus dexamethasone (Sigma, both 10^{-7} M) at day 7, 14 or 21. Lines were restimulated every 7 days.

Total CD4 experiments

Cells were cultured with 1α, 25-dihydroxyvitamin D3 (Biomol) or dexamethasone (Sigma, both 10^{-7} M) at day 0 in some of the total CD4 experiments. For TLR-2 stimulation experiments, Pam3cys (Invivogen) was added to cultures at day 0 at 5µg/ml.

2.2 Identification of Vβ receptor lineage

Th2 cells were cultured without or with 1α, 25-dihydroxyvitamin D3 and dexamethasone as above. Cells were then labelled with antibodies from the IOTest® Beta Mark kit (Immunotech) in accordance with the manufacturer's instructions. The Vβ receptor lineage was analysed by flow cytometry (FACSCalibur, BD). These experiments were kindly performed by Dave Richards (Hawrylowicz lab).

2.3 Carboxy-fluorescein diacetate succinimidyl ester (CFSE) labelling

CFSE labelling was performed using Cell Trace CFSE Cell Proliferation from Molecular Probes. Cells were washed twice in 1x PBS at 200g for 10 minutes at 4ºC and counted. Cells were labelled with 300µl of CFSE at a final concentration of 5mM diluted in 2.7ml PBS per sample, for 7 minutes at room temperature. Samples were then washed 3 times in 10% FCS/RPMI at 200g for 10 minutes at 4ºC. CFSE staining was confirmed by FACS analysis.

2.4 Functional experiments

Th2 cells were cultured for 28 days, with or without the addition of drugs at day 14 of culture. At day 28, cell lines were harvested, washed twice in 2%FCS HBSS (200g for 10 minutes at 4°C) and resuspended at $1x10^6$ cells/ml in 10%FCS/RPMI. Fresh autologous CD4+ T cells were isolated

using magnetic beads (Invitrogen) as previously described and then labelled with CFSE dye.

Effector Th2 cells or the putatively deviated Th2 cells (i.e. cells treated with calcitriol, dexamethasone and IL-10) were then co-cultured on 96 well plates with CFSE-labelled CD4+ T cells at various ratios in the presence of autologous APCs (T cell depleted PBMC and αCD3). Cultures were performed with or without anti–IL-10 receptor(5μg/ml), anti–TGF-β (5 μg/ml), anti-CTLA4 (5μg/ml) (all R&D systems, Abingdon, UK) or isotype control rat IgG2a (5μg/ml) (BD Biosciences) . Proliferation was determined by assessing the intensity of CFSE signal in the CD4 cells using flow cytometry (BD FACSCalibur).

2.5 Cytokine analysis by intracellular cytokine staining

Cells were fixed using 500μL BD Cytofix® (BD Biosciences) per condition for 30 minutes on ice followed by a wash (200g for 10 minutes at 4ºC) with 500μL 1x BD Perm/Wash® (BD Biosciences). Cells were counted and re-suspended at $2.5x10^6$cells/ml in Perm/Wash® before staining. 100μl samples ($2.5x10^5$cells) were stained with specific monoclonal antibodies directly conjugated to FITC, PE or APC fluorochromes for 30 minutes at room temperature in the dark. The samples were washed with Perm/Wash® followed by a second wash with BDFACSFlow® (BD Biosciences) (both at 200g for 10 minutes at 4ºC). After which, the samples were re-suspended with 500μL BDFACSFlow®. Matched isotype control antibody staining (BD Biosciences) was performed in parallel in each experiment. Samples were analysed on a FACSCalibur flow cytometer, following compensation, using CellQuest software version 4.0.2 (BD Biosciences). 10,000 live cells were analysed for fluorescence based upon forward and side scatter gating. Quadrant markers were set according to background staining of matched isotype control antibodies.

2.6 Cytokine analysis by ELISA

96 well plates were coated overnight with 1μg/ml specific catch antibody in bicarbonate buffer [0.1M, pH9.7] at 4ºC. The plates were washed four times with PBS buffer [PBS, 0.05% Tween-20 (Sigma-Aldrich). The relevant cytokine standards (rIL-5, rIL-10, rIL-13, rIFNγ, R&D systems) were added to the plates. Standards were prepared ranging from IL-5 – 20000pg/ml, IL-13 – 50000pg/ml, IL-10 –10000pg/ml and IFN-γ - 20000pg/ml with serial 1:10 dilutions in order to titrate a standard curve. The supernatant samples and standards in duplicate were also added to the plates, and the plates were incubated overnight at 4ºC. The plates were then washed four times in PBS/Tween 20 0.05% buffer biotin-labelled detection antibody for the appropriate cytokine was added to the individual plate at a concentration of 1μg/ml in detection buffer [PBS, 0.5% mouse serum (Sigma-Aldrich), 0.5% Tween-20, pH 7.4] at room temperature for 2 hours. The plates were washed a further four times and then ExtrAvidin® alkaline phosphatase enzyme (Sigma-Aldrich) was diluted 1/5000 in detection buffer and added to the plates at room temperature for 30mins. The ELISA was performed using matched antibody pairs (BD Biosciences; IL-5 (capture clone TRFK5; biotinylated detection clone JES-5A10), IL-10 (capture clone JES3-9D7; biotinylated detection clone JES3-12G8), IL-13 (capture clone JES10-5A2; biotinylated detection clone B69-2) and IFN-γ (capture clone NIB42; biotinylated detection clone 4S.B3). The IL-17 and IL-22 matched antibody pairs were obtained by using the IL-17 Duoset and IL-17 Duoset kits respectively as per the manufacturer's instructions (R and D systems).

Each plate was washed six times at the end of the ELISA process and phospho-nitrophenylphosphate substrate (Sigma-Aldrich) was added at 1mg/ml in Diethanolamine buffer [0.1M Diethanolamine buffer, pH9.8] to allow visualisation of cytokine concentrations. The levels of the individual cytokines were measured using a Fusion Plate reader (Packard, UK) at 405nm and quantified using Fusion Data analysis software version 1.60.0. The lower

limits of detection for assay of IL-10, IL-13, IFN-γ, and IL-5 were 50pg/ml, 100pg/ml, 50pg/ml and 100pg/ml, respectively.

2.7 IL-10 secretion assay

Isolation of live IL-10 secreting cells was performed using an IL-10 Secretion Assay Detection Kit (Miltenyl Biotec GmbH, Gladbach, Germany). Cell lines were harvested at day 28, washed twice in 2%FCS HBSS and resuspended at $1x10^6$ cells/ml in 10%FCS/RPMI. Cells were then restimulated with anti-CD3 (1µg/ml) and 50U/ml rIL-2 for 16 hours at 37°C/5%CO2. Cells were then collected and washed in ice-cold selection buffer [PBS (Life Technologies), 0.5% FCS, 2mM EDTA (Sigma-Aldrich), pH7.4] at 200g for 10 minutes at 4°C. The supernatants from samples were removed by pipetting and cell pellets were resuspended in 80µl ice-cold 10%FCS/RPMI per 10^7 cells. 20µl of IL-10 catch reagent (Miltenyl Biotec) was added per 10^7 cells and the samples were incubated for 5 minutes on ice.

After the 5 minute incubation, 10%FCS/RPMI was added to give a cell density of 1 x 10^7 cells/ml and the samples were incubated under continuous rotation for 45 minutes at 37°C/5%CO2 to allow secretion of IL-10. Samples were then placed briefly on ice and washed in ice–cold selection buffer (200g for 10 minutes at 4°C). The supernatants from samples were removed by pipetting and the cell pellets resuspended in 80µl ice-cold selection buffer per 10^7 cells 20µl of PE-conjugated IL-10 antibody was added per 10^7 cells. Any addition staining reagents such as FITC conjugated CD25 (clone M-A251); 10µl per 10^7 cells; BD biosciences) were added and the samples incubated on ice for 10 minutes. Cells were washed for a final time in ice-cold selection buffer and resuspended in 2%FCS/HBSS prior to flow cytometry.

2.8 Cytokine bead array analysis of cytokines

ELISA analysis was complemented by the use of the cytokine bead array for the functional experiments. The human Th1/Th2/Th17 kit (BD biosciences)

was used for analysis as per the manufacturer's instructions. Samples from the assay then run on a BD LSR II flow cytometer and quantified using BD FCAP array software. The lower limit of detection was 1.5pg/ml.

2.9 FoxP3 intranuclear staining

FoxP3 staining was performed with a PE- conjugated FoxP3 antibody and the FoxP3 staining buffer set, (Ebiosciences, San Diego, USA) according to manufacturer's instructions. Briefly, $1x10^5$ cells were stained for surface expression of CD4 and CD25 or the matched isotype control antibody as detailed in section 2.6. After which, the cells were washed twice in ice-cold FACS flow (200g at 4°C for 5 minutes). The cell pellet was resuspended by pulse vortex, 500µl of 1x Fixation/Permeabilisation solution added to each sample and incubated for 30 minutes at 4°C in the dark. The samples were washed twice in 1x Permeabilisation buffer (1ml; 200g for 5 minutes at 4°C). 7µl (87.5ng; optimised by titration) of PE- or APC- conjugated FoxP3 antibody (clone PCH101) or 87.5ng of rat IgG2a control antibody was added to the appropriate tube in 1x Permeabilisation buffer and the samples were incubated for 30 minutes at 4°C in the dark. Following two final washes in permeabilisation buffer, the cells were resuspended in 250µl FACS flow prior to FACS analysis. Quadrant markers were set according to the matched isotype control antibody staining.

2.10 IL-13 secretion assay

Isolation of live IL-13 secreting cells was performed using an IL-13 Secretion Assay Detection Kit (Miltenyi Biotec GmbH, Gladbach, Germany). CD4+ cells from atopic patients were isolated by magnetic bead selection as previously, washed twice in 2%FCS/HBSS and resuspended at $1x10^6$ cells/ml in 10%FCS/RPMI with 5% human serum to minimize background staining. Cells were then restimulated with anti-CD3 (1µg/ml) and 50U/ml rIL-2 for 16 hours at 37°C/5%CO_2. Cells were then labelled with IL-13 catch reagent at 20µL per 10^7 cells (5 minutes on ice), and incubated for 45 minutes at 37°C. Cells were then labelled with IL-13 detection biotin antibody at 20µl per 10^7 cells (10 mins

on ice) and washed with cold buffer. They were resuspended in cold buffer and labelled with anti-biotin PE (20µL per 10^7 cells). Cells were washed again in cold buffer and resuspended in 1ml HBSS. Unlabelled cells were used as a negative control for compensation. IL-13 PE was added to unlabelled cells after the secretion step as a positive control. Cells were labelled with CD4 FITC antibody (as previously) and sorted according to IL-13 secretion using a FACSAria cell sorter (see main text for purity).

2.11. Quantification of mRNA expression

2.11.1 RNA extraction and quantification

Total RNA was isolated from 'snap frozen' cell pellets (frozen in liquid nitrogen and stored at -80°C) using the Qiagen RnEasy mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturers instructions. RNA was treated with Raze-free DBase (Qiagen) before being re-purified using the Resay kit and eluted into 30µl of nuclease-free H_20. RNA was quantified with a Nano Drop ND-1000 spectrophotometer using ND-1000 software version 3.2.0. The purity and integrity of the RNA was assessed by A_{260}/A_{280} spectrophotometric measurements.

2.11.2 Reverse Transcription

Typically, 250ng RNA was reverse-transcribed into complimentary deoxyribonucleic acid (cDNA) in a 30µl reaction. 0.2µg random hexamers (Amersham Biosciences, Buckinghamshire, UK) and 250ng RNA were heated to 70°C for 5 minutes, and allowed to cool on ice. A master mix containing a final concentration of 1x reverse transcriptase reaction buffer (MBI Fermentas, Sunderland, UK), 1mM 4dNTP (MBI Fermentas), 20U RNAguard® was added to each sample and the reactions were incubated at 25°C for 5 minutes. 200U of Revertaid Mouse Moloney Leukaemia Virus (M- MuLV) reverse-transcriptase enzyme (MBI Fermentas) was added per reaction. The samples were incubated at 25°C for 10 minutes, 42°C for 10 minutes, 70°C for 10

minutes and held at 4°C. The cDNA generated was stored at -20°C until subsequent analysis by Real time RT-PCR.

2.11.3 Real time RT-PCR

Primer and Probe sets

Transcriptional expression of target mRNA transcripts were determined by PCR amplification, quantified by 5'-nuclease assay using fluorescent labelled TaqMan probes and analysed using an ABI PRISM 7900HT Sequence Detection System thermal cycler (Applied Biosystems, Foster City, USA). All primers/probes sets were purchased from Applied Biosystems. The probes have a fluorescently labelled reporter dye, either VIC or FAM that is covalently linked to the 5' -end and a downstream quencher that is not labelled with fluorescent dye. The Real time RT-PCRs were internally controlled using primers and probes for 18S ribosomal RNA. The 18S probe had a VIC reporter dye, whereas all of the other probes had a FAM reporter dye. These distinguishable reporter dyes allowed amplification of 18S rRNA and the target gene of interest in a multiplex reaction. The RT-PCR was performed in a total volume of 10µl per reaction. The Master Mix contained 10ng reverse transcribed RNA, 0.5x gene specific probe set and 0.5x 18s probe set and each sample was performed in triplicate. The cycle parameters were: 50°C for 2 minutes; 95°C for 10 minutes for 1 cycle followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

2.11.4 -(ΔΔCt) analysis

For each sample the threshold cycle (Ct) was determined for the target gene and the endogenous control (18s), using SDS software version 2.1 (Applied Biosystems). The data were first normalised by subtracting the Ct value of the endogenous control from the target gene (Δ Ct). The Δ Ct of the neutral condition sample (control sample) was subtracted from the Δ Ct of all other samples to give Δ Δ Ct. Finally, the sample mRNA abundance was calculated by the formula $2^{-(\Delta\Delta Ct)}$.

2.2. Exon ST microarray analysis

Experiments for the Exon ST microarray analysis were kindly performed with the assistance of the KCL Genomics centre, Waterloo.

mRNA extracted from the Th2 cell lines and deviated cell lines of interest (extracted as described above) were analysed for RNA integrity using the bioanalyser and also quantified using the nanodrop. Samples listed were as follows. See main text for RNA analysis.

Table 2 mRNA samples for exonST microarray

Sample no	Experiment no	Condition	Day of culture prior to isolation
1	34	Th2	21
2	70	Th2 VDIL-10	28
3	34	Th2 IL-10	28
4	34	Th2 IL-10	28
5	34	Th2 VD	28
6	34	Th2	28
7	70	Th2	28
8	70	Th2	21
9	70	Th2 VD	28
10	70	Th2 IL-10	28
11	69	Th2 IL-10	28
12	37	Th2 VD	28
13	37	Th2 IL-10	28
14	37	Th2 VDIL-10	28
15	37	Th2	28
16	69	Th2	21
17	69	Th2 VD	28
18	69	Th2	28
19	69	Th2 VDIL-10	28

3 samples from 4 different cell lines were pooled to represent appropriate conditions with a 5th set used to facilitate normalisation (d21 samples). cRNA and cDNA was prepared from the samples using the Ambion® WT Expression Kit (Affymetrix, CA) and the cDNA was subsequently fragmented, labelled and hybridised to the Exon ST gene chips using the Affymetrix

GeneChip WT Terminal Labeling Kit (Affymetrix, CA). One exon ST cell chip was used for each condition.

Analysis of the Exon ST gene chip array was performed using the Affymetrix Expression Console Software analysis to generate mean intensity values after normalisation and broadly assess fold changes between cells. It was not possible to do further computational analysis due to pooling of the samples.

2.3. Statistical Analysis

Statistical analysis was performed using Prism 5 for Mac OS X. Data are represented as arithmetic means ± standard error means (SEM). A student's t test was used to test the Null Hypothesis and determine differences between two sets of data. One-Way ANOVA was used to compare more than two groups of data. Differences were considered if p was <0.05.

Chapter 3

The deviation of effector Th2 cells towards a regulatory phenotype

3.1 Introduction

The Th2 cell is a pivotal cell in the pathogenesis of allergic airway disease[133] characterised by expression of the transcription factor GATA3[134] and secretion of the cytokines IL-4, IL-13 and IL-5[71]. The majority of treatments aimed at decreasing the allergic response have been devised with the aim of blocking or counteracting further production of Th2 related effector cytokines and their associated pathological effects. Patients with asthma have previously been categorised into Th2 high or Th2 low subtypes, with a Th2 high phenotype denoting eosinophilia and steroid sensitivity in some cases. There remains a substantial proportion that does not exhibit complete responsiveness to this treatment with resultant morbidity and mortality. Further work on phenotypes suggests that Th17 cells may coexist with Th2 cells and promote disease progression.

Strategies to target the effects of pathogenic effector cytokines may involve directly blocking or reducing levels of the effector cytokine(s) as outlined above. Conversely, an alternative strategy would involve indirect methods of blocking the pathogenic response by increasing the number of cells or cytokines that will counteract the effector response. Regulatory T cells could provide this counterbalance.

Patients with allergic disease are known to have deficiencies in their natural Foxp3+ T reg profile[98], which can inhibit via various immune mechanisms including inhibitory receptors such as CTLA4, competition for essential growth factors and inhibitory cytokines and TGFβ[23]. In addition, Foxp3- T cells secreting the anti-inflammatory cytokine IL-10, also appear impaired[135] in patients. Allergen-reactive CD4+ IL-10-secreting T cells have been shown to be present in higher frequencies in cultures from non-allergic as compared to allergic individuals (who have higher frequencies of allergen-responsive IL-4+ T cells)[136]. Therefore strategies that will enhance IL-10 levels and peripherally induced IL-10 Treg function are generally perceived to be advantageous[137]. Therapy involving adoptive transfer of Tregs would appear to be a method of

enhancing this cell population, but this work is at a relatively early stage, impractical for the treatment of allergic disease and not yet ready for the clinic[138]. Recent evidence suggests that the Th1 and Th2 phenotype may not represent terminal states of T cell differentiation[21]. Th2 cells have the capacity to change phenotype to produce non-Th2 effector cytokines, but there is also evidence to suggest that a variety of CD4+ T cells with an effector phenotype have the capacity to express IL-10, which can damp down the initial effector response[139]. Furthermore, this phenomenon may lead to the development of tolerance, and also represent the transformation of effector cells towards regulatory cell phenotypes.

There is evidence that this phenomenon of tolerance occurs naturally in subjects who are tolerant to antigens as a result of natural exposure; bee-keepers who undergo repeated exposure to bee sting venom develop decreased early systemic responses to the allergen, associated with decreased frequency of phospholipase-A specific IL-4+ CD4+ T cells and an increase in phospholipase-A specific IL-10+ CD4+ T cells[140]. Tolerance may also be achieved through administration of immunotherapy, by suppression of Th2 responses and promotion of regulatory cell responses[141]. Redirection of responses in these cases appears to be associated with IL-10 production.

Stimulation of freshly isolated peripheral blood CD4+ T cells together with the active form of vitamin D, calcitriol, and dexamethasone, has been shown to increase the frequency of IL-10+ T cells *in vitro*, both in mice and in humans [42,131,142]. The secretion and action of IL-10 is enhanced by the addition of exogenous IL-10 to culture[42].

Hypothesis

Effector Th2 cells can be deviated towards a regulatory phenotype using the active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1α,25$(OH)_2$D3, or calcitriol) and dexamethasone in culture. Furthermore, the phenotype of the regulatory cells will be comparable to previously described IL-10+ Tr1 T regulatory cells, given the propensity of these drugs to promote an IL-10 phenotype[131].

Experimental aims

- To establish a culture system to reproducibly generate human Th2 cell lines

- To use this methodology to investigate the immunomodulatory effects of calcitriol, dexamethasone, and IL-10 to deviate this Th2 response towards an immunoregulatory IL-10+ Tr1 profile, assess the function of the deviated cells in a standard *in vitro* immune suppression assay, and assess the phenotype of cells for expression of Th2 and Treg associated transcription factors.

3.2 Results

3.2.1 Generation of effector Th2 cell lines from peripheral blood naïve CD4+ T cells

Cell lines were generated using a modified departmental protocol[143] utilising naïve CD4+ T cells isolated from peripheral blood mononuclear cells (Figure 3.1). The protocol involved TCR stimulation with anti-CD3 and co-stimulation with anti-CD28 without the use of antigen presenting cells. Th1 responses were blocked by the use of anti-IL-12 in the first week, followed by continued culture in the presence of an anti-IFNγ antibody. Th2 responses were promoted by the use of IL-4. An anti-IL-10 antibody was not used in the modified protocol as IL-10 production needed to be assessed both in the cell line and the deviated cells. Cell lines were restimulated weekly with anti-CD3 and anti-CD28. IL-2 was present throughout the whole duration of the culture.

The Th2 phenotype was assessed using intracellular cytokine staining. A representative plot of the cell line is shown (Figure 3.2A) with analysis performed after three rounds of stimulation, i.e. on day 28 of culture. PMA and ionomycin stimulation of the T cell lines for 2 hours prior to intracellular cytokine staining induced a high frequency of IL-13 positive cells when analysed by flow cytometry. In this example almost 60% of cells were IL-13 positive, whilst 23% of cells stained IL-5 positive. This was confirmed on analysis of mean data for cytokine production in 3 individual experiments (Figure 3.2B). Reassuringly, there was a low level of IFNγ detected after repeated rounds of stimulation (Figure 3.2A and data not shown), demonstrating the efficacy of Th1 cytokine blockade.

Figure 3.1. Schematic diagram representing protocol for generation of Th2 cells

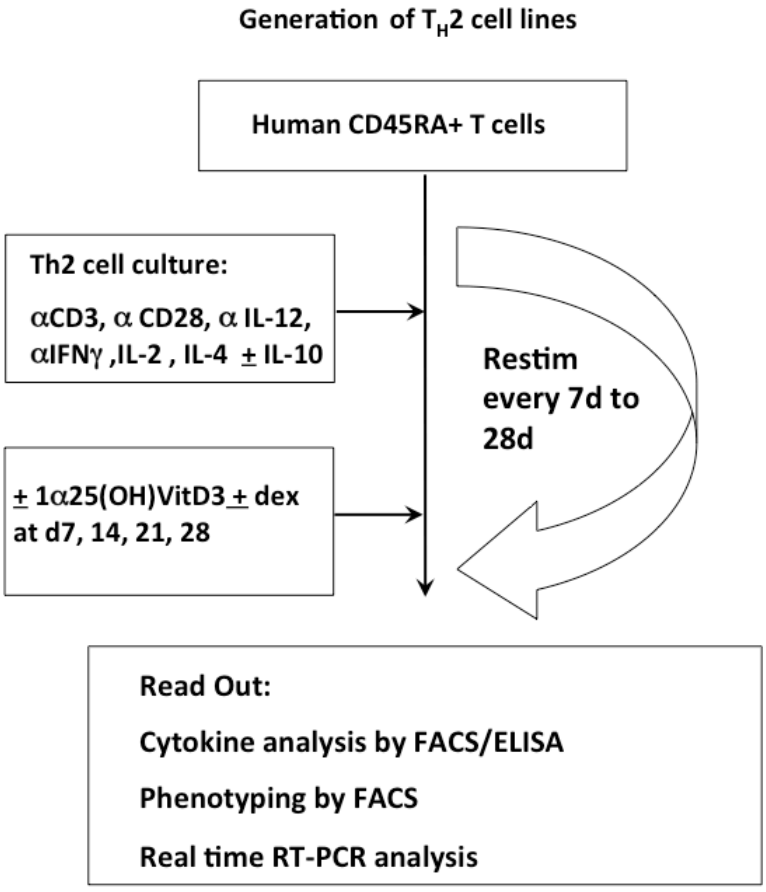
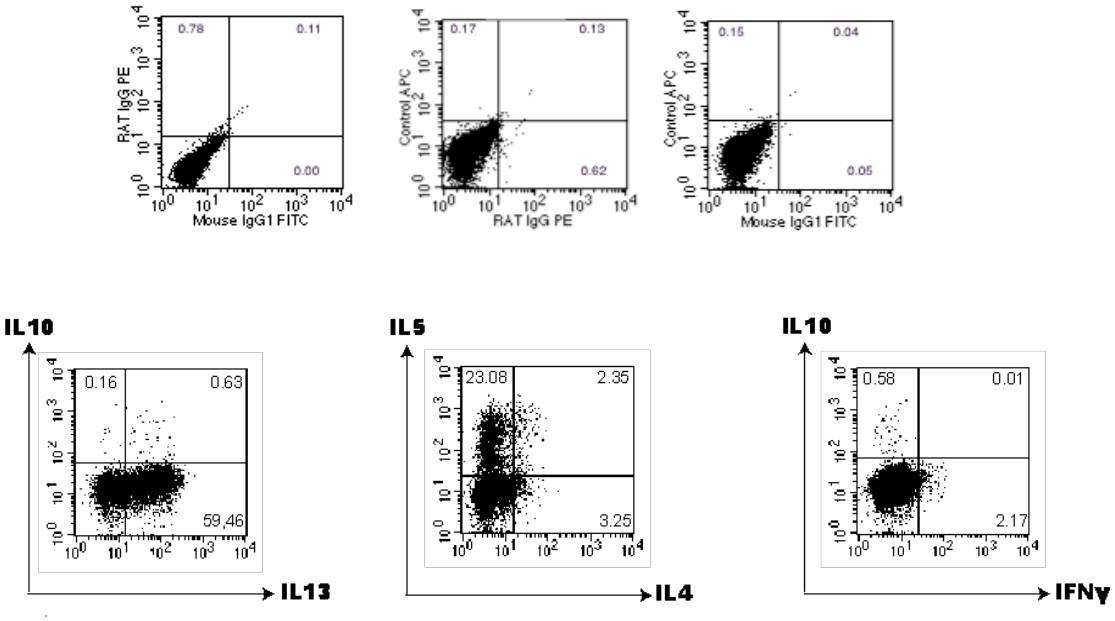


Figure 3.2 Generation of Th2 cell lines

A



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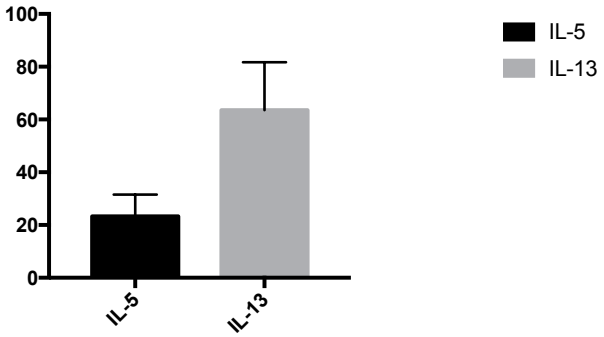


Figure 3.2A Flow cytometry analysis of Th2 cell lines. Please refer to figure 3.1 and materials and methods for the protocol. Intracellular cytokine staining confirmed the Th2 phenotype of the cell line, demonstrating a high frequency of IL-13 and IL-5 positive T cells, and a low frequency of IFN γ positive and IL-10 positive T cells by day 28 of culture.

Figure 3.2B Mean cytokine production of Th2 cell lines. Th2 cell lines were cultured as per protocol in fig A and intracellular cytokine staining was performed as above. Mean cytokine production for Th2 cytokines IL- 5 and IL -13 at day 28 is shown (n=3 experiments), p = NS, t-test.

3.2.2 Deviation of effector Th2 cells towards a regulatory phenotype occurs in the presence of 1α,25[OH]$_2$ vitamin D3 and dexamethasone

Previous work performed by this laboratory group has demonstrated the capacity of calcitriol and dexamethasone to deviate CD4 cell lines towards an IL-10+ve Treg phenotype[131]. The Th2 cell lines that were established in this set of experiments were further interrogated by the addition of calcitriol and dexamethasone, to establish if the effector cell lines could be deviated away from the effector phenotype. Calcitriol and dexamethasone were both added at a dose of 10^{-7} M as this has been shown to promote high levels of IL-10 secretion in freshly isolated peripheral blood CD4 cell cultures based on extensive earlier work in our lab[39,42,131]. Cell lines were cultured with and without exogenous IL-10[42] at different doses from day 0 of culture (Figure 3.1) to assess any interaction or modification of response. The drugs calcitriol and dexamethasone were initially added at 7 days after the commencement of Th2 cultures, for the pilot set of experiments.

The deviated cells were assessed for cytokine production via intracellular cytokine staining (Figure 3.2). IL-13 production was diminished in the presence of either calcitriol or dexamethasone when added as single drugs (data not shown), but the reduction was most profound and reproducible with the combination of calcitriol and dexamethasone. This effect was strengthened by culture in the presence of IL-10 (Figures 3.3 and 3.4). This effect persisted through several rounds of stimulation in the continued presence of the drugs in culture. This was seen even with the addition of drugs at later time points (Figure 3.4 and 3.5).

Further deviation experiments were performed using drugs added at d14 so that a putatively more differentiated Th2 cell line had been established. Analysis of deviation at this time point corroborated the previous observation that Th2 cytokine production was diminished after addition of the drugs (Figure 3.4). Interestingly, and unexpectedly, IL-10 production did not significantly increase in the cell lines deviated with calcitriol and

dexamethasone alone. Significant levels of IL-10 production (by intracellular cytokine staining) were seen with the addition of exogenous IL-10 to the deviation cultures, in association with abrogation of IL-13 production (Figure 3.4). This finding contrasts with experiments involving culture of freshly isolated CD4+ T cells where addition of exogenous IL-10 was not required[131], but concurs with earlier work in animal studies demonstrating a requirement for exogenous IL-10 to promote IL-10 production in the presence of calcitriol and dexamethasone[42]. This is likely explained by the loss of IL-10 synthesis by Th2 cells as they become more differentiated (see Figure 3.4).

The development of an IL-10 response is not instantaneous and it can take up to 14 days to demonstrate appreciable levels of IL-10 *in vitro* (Figure 3.4). The IL-10 production represents true secretion rather than a reflection of the exogenous IL-10 added to culture, as the levels demonstrated by CBA and intracellular cytokine staining were performed after washing and restimulation of the deviated cells (Figure 3.4).

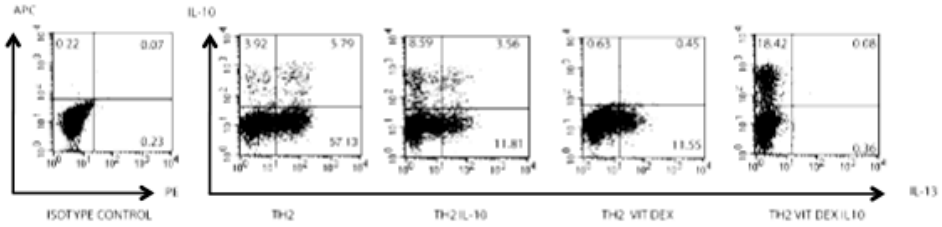
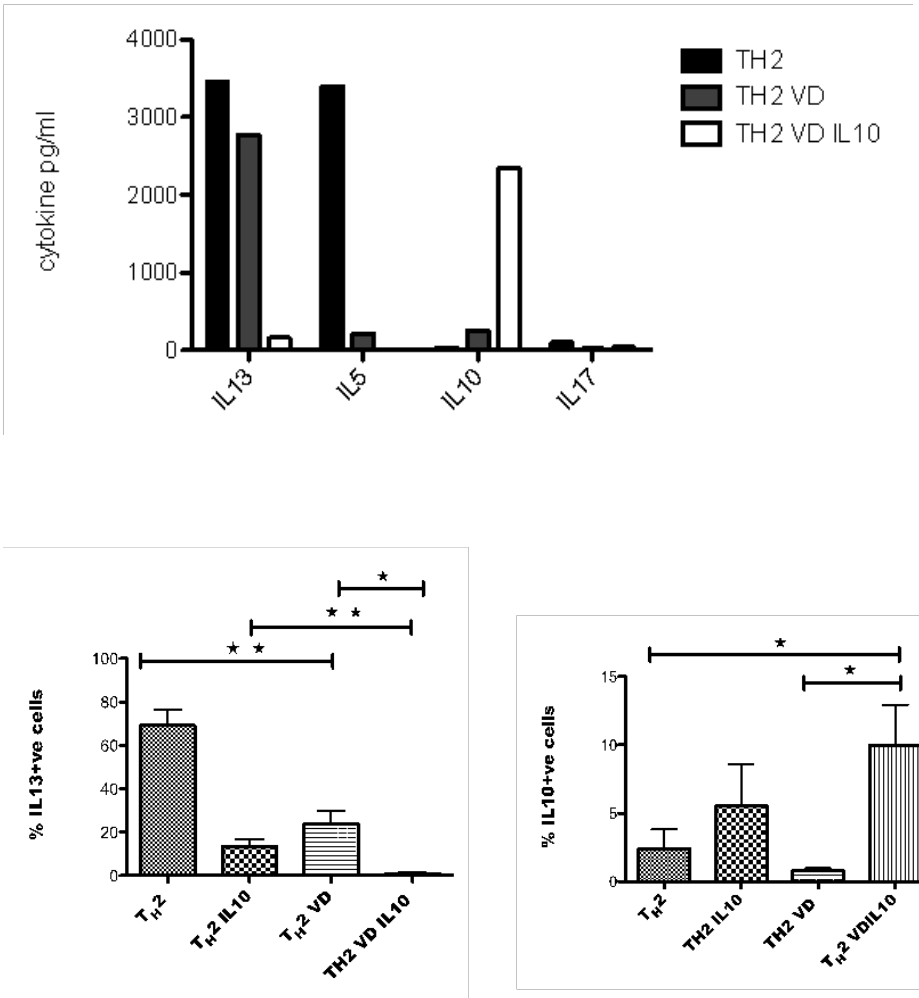


Figure 3.3 Deviation of Th2 cell lines in the presence of 1α,25 $[OH]_2$ vit D3, dexamethasone and IL-10

Naïve CD4+ T cells were restimulated weekly under standard Th2 conditions (Th2), some in the presence of IL-10 (Th2 IL-10) or with addition of 1α,25$[OH]_2$ D3 plus dexamethasone (both at 10-7 M) from day 7 of culture. The addition of exogenous IL-10 inhibits cytokine production and promotes IL-10 synthesis. 1α,25$(OH)_2$D3 and dexamethasone deviates Th2 cells with a reduction in Th2 cytokines at day 21, after two rounds of stimulation. Representative flow cytometry plots.

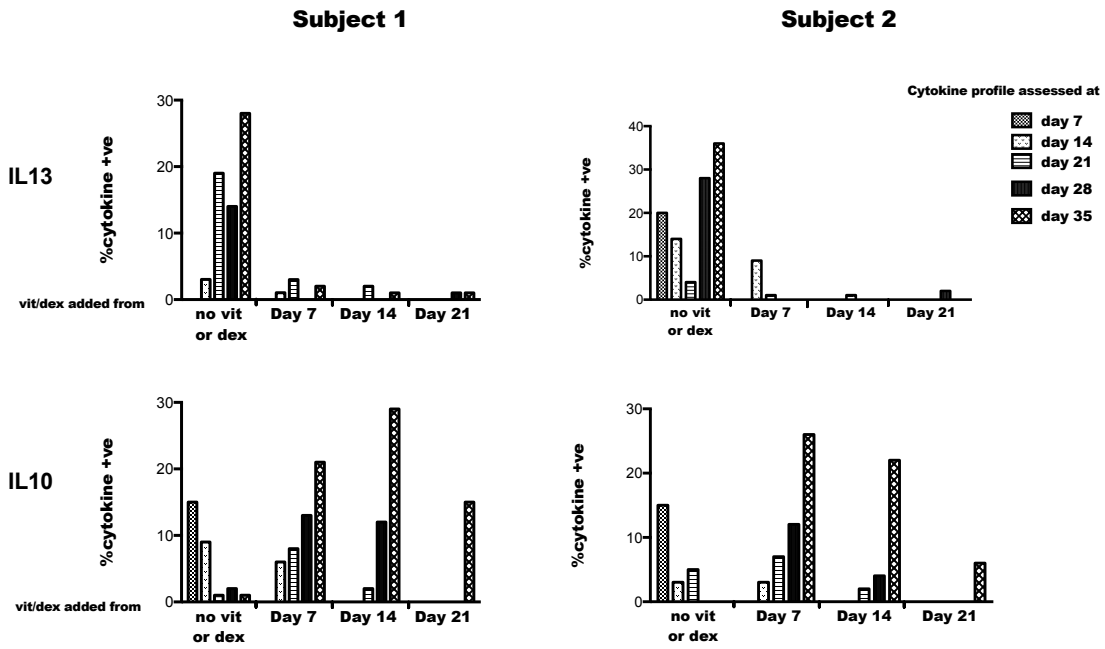
Figure 3.4 Deviation of Th2 cells



Top panel . Naïve CD4+ T cells were restimulated weekly under standard Th2 conditions (Th2), some in the presence of IL-10 (Th2 IL-10) or with addition of $1\alpha,25[OH]_2$ D3 plus dexamethasone (both at 10^{-7} M) from day 14 of culture. Supernatants were extracted from cell culture at day 28 and analysed by cytokine bead array. Figure 3.4A shows an experiment from an individual donor (n=1)

Bottom panel. Naïve CD4+ T cells were restimulated weekly under standard Th2 conditions (Th2), some in the presence of IL-10 added from day 0 (Th2 IL-10) or with addition of $1\alpha,25[OH]_2$ D3 plus dexamethasone (both at 10^{-7} M) from day 7 of culture. Figure shows mean values of intracellular cytokine staining performed after PMA and ionomycin stimulation at day 28. Graph represents mean of 4 experiments with 4 individual donors + SEM. *p<0.05, ** p <0.01, one way ANOVA

Figure 3.5 Deviation can occur with addition of drugs added from day 14 and even as late as day 21 of Th2 cell line culture



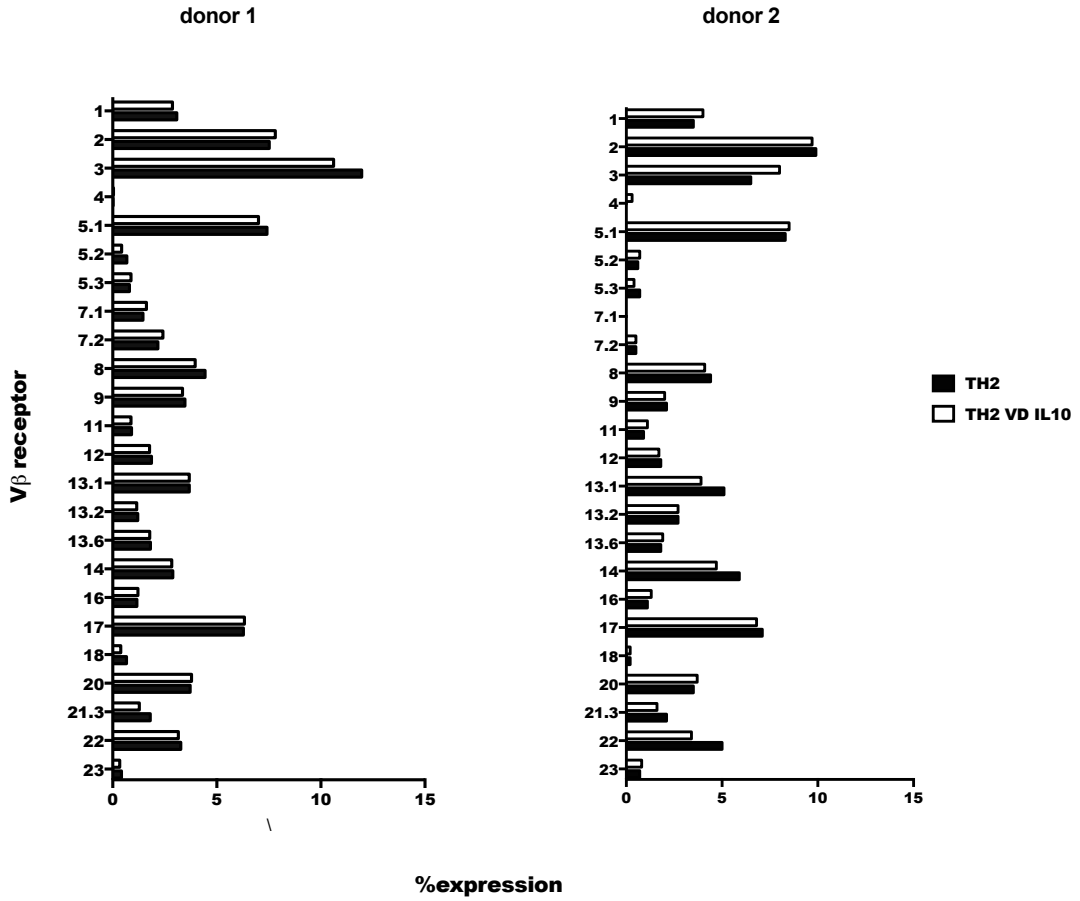
Th2 cells exhibit plasticity towards a regulatory phenotype even after several rounds of differentiation. CD4+ cell lines were cultured under Th2 conditions with IL-10 added at d0. Some Th2 cell lines were cultured in the presence of $1\alpha,25(OH)_2D3$, and dexamethasone which was added at different time points following the start of the culture from day 7, day 14 or day 21 (indicated on x axis). Cytokine profiles were analysed by flow cytometry at the time points indicated in the legend (shown in the top right hand side of figure). Representative data from two individual donors is shown.

3.2.3 TCR Vβ usage in the cell population does not alter upon deviation of the Th2 cells using calcitriol and dexamethasone

Experiments outlined in the results above demonstrated that Th2 cell lines can be deviated towards an IL-10+ve phenotype, but it was unclear if this represented a single or minor population that was being expanded in culture, or a true deviation manifested by polyclonal expansion. An experiment was therefore performed to assess the nature of TCR usage of both of the cell lines and the deviated population in order to explore this question in more detail. Th2 cell lines and Th2 cell lines cultured in the presence of IL-10 were subsequently deviated with the addition of drugs from d14 and then cultured for a total of 28 days. Cells were isolated at day 28 of culture and their TCR Vβ usage was assessed using a special fluorochrome staining kit, enabling analysis of Vβ receptor usage by flow cytometry.

Analysis by flow cytometry demonstrated a polyclonal distribution of expansion of T cell lines as demonstrated by the wide variety of expression of TCR Vβ receptor phenotypes seen in the cell lines at day 28 (Figure 3.6). There was no obvious change in Vβ receptor usage between the Th2 cell line and the deviated cell line with the same polyclonal spread of Vβ receptor usage. This finding indicates that the IL-10+ve cells seen in Figures 2 and 4 are unlikely to represent the outgrowth of a minor population of IL-10 producing T cells. The changes in cytokine profile may therefore represent deviation.

Figure 3.6 Vβ receptor lineage analysis of Th2 cell lines and deviated cells



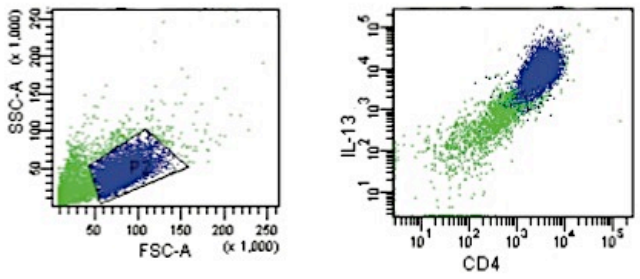
T_h2 cells were cultured without or with $1\alpha,25(OH)_2D3$, D3 and dexamethasone from day 14 of culture. The Vβ receptor lineage was analysed by flow cytometry at day 28. Figures represent data from two individual donors.

3.2.4 Isolation of peripheral blood IL-13+ cells *ex vivo* and response to deviation culture conditions

The initial deviation experiments were performed on cell lines generated from peripheral blood cells. To further test the hypothesis that effector Th2 cells could be deviated with the drugs, an extension of the experimental design was devised to test the concept in a more physiological context, using effector cells that have undergone development *in vivo* rather than extended culture *in vitro.* The experimental design incorporated isolation of IL-13 secreting cells that were only briefly stimulated in culture (<24hours), as a surrogate for established Th2 cells from a human donor.

PBMCs were isolated from human donors as previously described. CD4+ T cells were isolated using magnetic beads and a live cytokine secretion assay was performed to label IL-13 positive cells from freshly isolated peripheral CD4+ T cells following stimulation with anti-CD3 and IL-2 overnight only (as per manufacturers instructions). IL-13 +ve cells were isolated by FACS sorting (Figure 3.7).

Figure 3.7 Isolation of IL-13 +ve cells following secretion assay - purity



CD4+ cells were isolated from PBMCs *ex vivo* and stimulated overnight with αCD3 and IL-2. IL-13+ve cells were then isolated by FACS following an IL-13 secretion assay with > 95% IL-13 purity. Blue cells represent the IL-13+ve cells.

3.2.5 Results of *ex vivo* isolation and stimulation

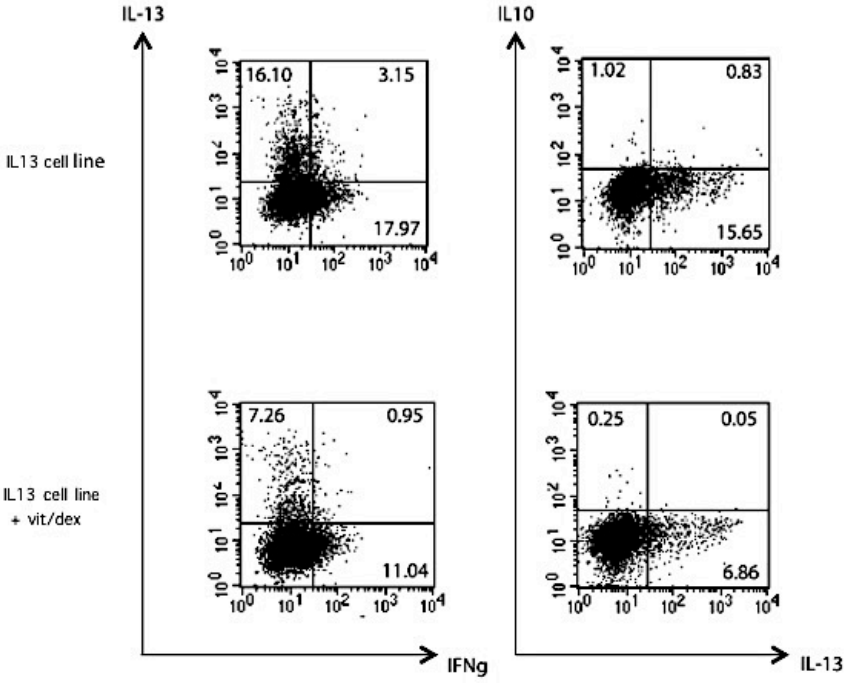
The number of cells isolated post sort was small (less than 100,000). Cell culture was therefore performed using 96 well plates rather than the 24 well plates previously used for the *in vitro* cell line experiments. The sorted cells were therefore stimulated with anti-CD3, IL-2 and IL-4 every 7 days to allow expansion to occur. Cell numbers obtained during the earlier rounds of stimulation were too low to enable the cultures to be split (the initial plan had been to leave one group of the *ex vivo* cells for simple restimulation and another for deviation). This change in experimental design meant that calcitriol and dexamethasone were not added until day 28. A representative plot of the deviation experiments is shown in Figure 3.8.

The percentage of cells that were still positive for IL-13 by intracellular cytokine staining at day 28 was approximately 16%, (which may reflect the earlier problems with identification of the true IL-13+ve cells in the context of non-specific binding, see section 2.10 in Materials and Methods). Nevertheless, addition of calcitriol and dexamethasone at day 28 did induce a downward trend in the number of IL-13+ve cells (by about half). This did not correlate with an increase in IL-10 expression. This probably reflects the fact that culture conditions did not include exogenous IL-10.

It was therefore decided that this line of experimental design would not allow us to address our original question. Low yields of IL-13+ T cells obtained directly *ex vivo* were insufficient to allow the drugs to be added immediately to culture. Expansion of effector cells meant that we were no longer studying an *ex vivo* Th2 cell, and in any case still did not enable sufficient numbers of cells for genetic analysis with mRNA.

The remaining experiments were carried out using the Th2 cell lines generated *in vitro* and the cell lines deviated by the addition of drugs.

Figure 3.8 Assessment of cytokine production following cell culture of IL-13+ cells isolated *exvivo*



CD4+ cells were isolated from PBMCs *ex vivo* and stimulated overnight with αCD3 and IL-2. IL-13+ve cells were then isolated by FACS following an IL-13 secretion assay > 95% IL-13 purity. Cells were then cultured in the presence of IL-2 and IL-4 and restimulated every 7 days with αCD3. 1,25$(OH)_2$D3, and dexamethasone were added at d28. Intracellular staining was performed at d35. Figure 3.8representative of 2 experiments.

3.2.6 Deviated Th2 cells (Th2 IL-10 VD cells) have suppressive function, which is not abrogated in the presence of anti IL-10 receptor antibody

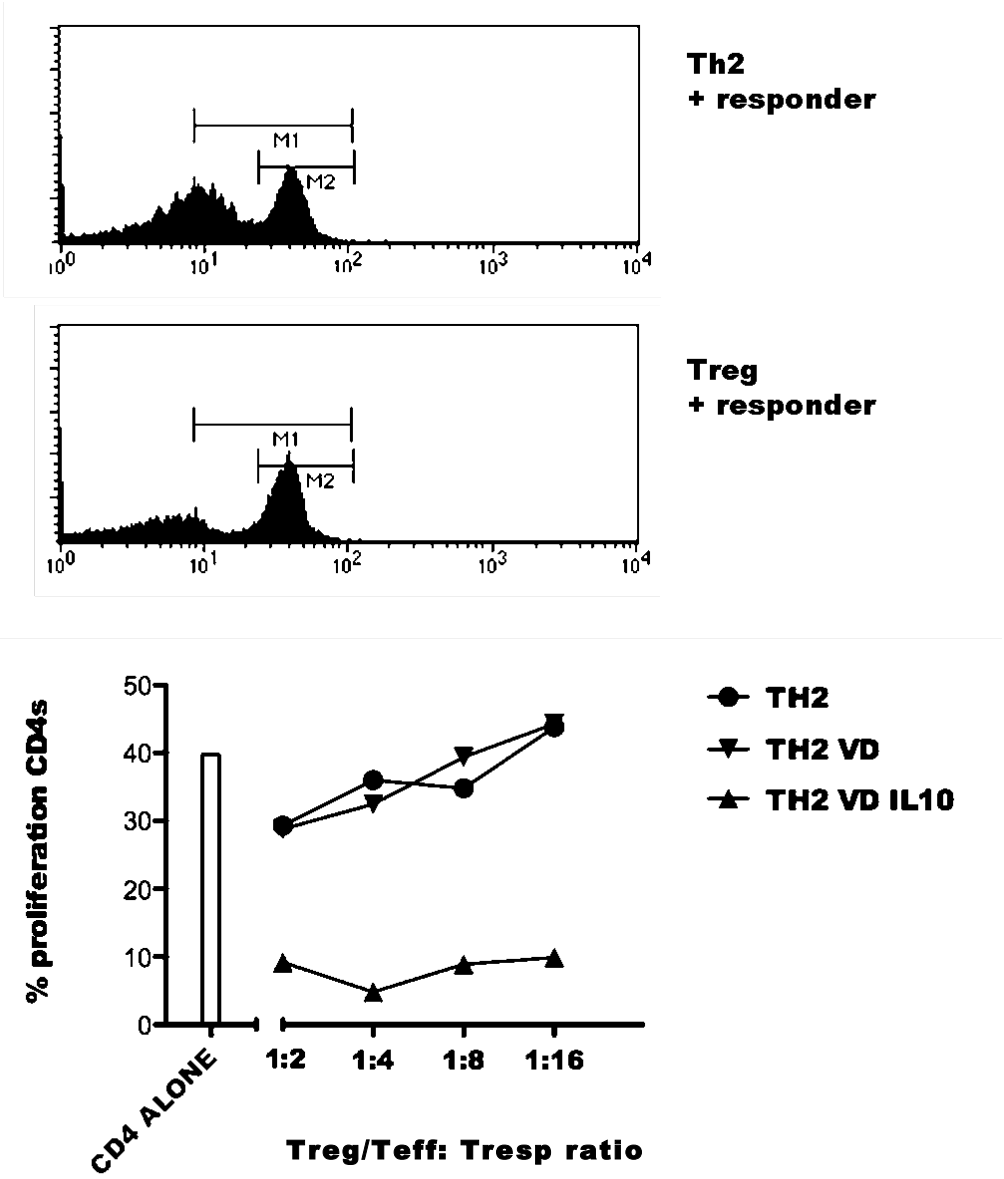
The experiments described above demonstrate the capacity of Th2 cell lines to undergo deviation towards a putative regulatory cell profile *in vitro*, with persistence of the phenotype for at least 14 days, and no evidence to suggest reversion to secretion of the effector cytokines. Further experiments were performed to ascertain if the deviated cells were able to suppress the

proliferation of autologous CD4s, and therefore had the potential to inhibit inflammatory responses.

Cells were isolated at d28 following 14 days of treatment with drugs. Fresh autologous CD4s were also isolated at the same time point and labelled with CFSE, a dye that allows analysis of cell proliferation by flow cytometry. The freshly isolated CFSE-labelled CD4s were then co-cultured with the cell lines at the indicated ratios in the presence of autologous APCs and αCD3.

Proliferation assays demonstrated that the baseline level of proliferation when the autologous CD4 cells were stimulated alone with αCD3 plus autologous APCs was approximately 40%. This level of proliferation was maintained when the autologous T cells were co-cultured with Th2 cell lines. As expected, the Th2 effector cell lines, when isolated at day 28 and co-cultured with fresh autologous CD4s did not demonstrate inhibition of proliferation. (Figure 3.9). When the CD4s were cultured in the presence of the Th2 cells that had been deviated with calcitriol, dexamethasone and IL-10 there was suppression of proliferation. This suppression was seen with cell ratios as low as 1 suppressor cell to 16 CD4s (Figure 3.9.). Co-culture of cells deviated with vitamin D and dexamethasone alone (i.e. not cultured with exogenous IL-10) and autologous CD4s surprisingly did not result in any inhibition of proliferation. The Th2 + IL-10 condition was not tested.

Figure 3.9 Analysis of regulatory function of deviated cell line by CFSE



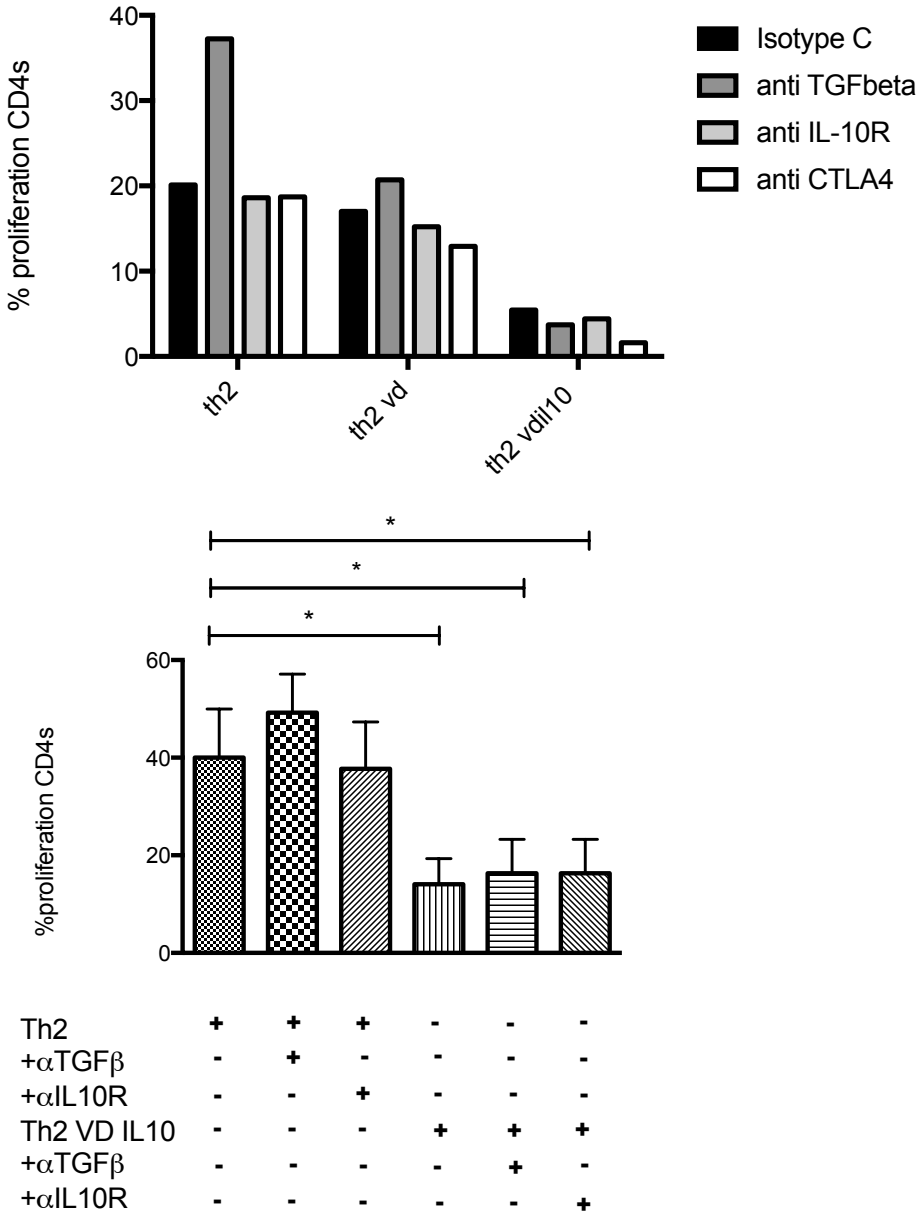
Th2 cell lines and deviated T cell lines were assessed by co-culture with autologous, freshly isolated, CFSE-labelled CD4+ T cells in the presence of autologous APCs. Top figure, representative CFSE plots from one of three experiments showing co-cultures using Th2 cell line and Treg cell line. Cultures were performed at varying ratios of deviated (Treg) or effector (Teff) cells to responder CD4 cells (Tresp). Bottom figure. Following 72hrs of stimulation, proliferation was assessed by intensity of CFSE signal as measured by flow cytometry. Approximately 40% of the CFSE-CD4+ responder T cells proliferated (n=1 experiment with titration to 1:16). Th2 cells deviated by addition of dexamethasone, $1\alpha,25(OH)_2D3$, and IL-10 to culture caused profound inhibition of CD4+ responder T cell proliferation at all ratios tested.

Co-cultures were also performed in the presence of blocking antibodies to evaluate if suppression was related to IL-10 synthesised by the deviated Th2 cells (Th2 VD10), or TGFβ, another suppressive cytokine involved in regulatory T cell function. Suppression of proliferation by the drug deviated cells was not affected by blockade of TGFβ (Figure 3.10). Surprisingly, proliferation of CD4s was not restored by the presence of anti-IL-10 receptor antibody either. There was also no change in proliferation when anti-CTLA 4 antibody was added to the co-culture, although CTLA4 is increased in freshly isolated peripheral blood CD4+ T cells following culture with vitamin D[144].

The result of the mean proliferation data for co-cultures performed in the presence of anti-IL-10 receptor antibody was unexpected, given that the blocking antibody is a reagent that has been previously utilised in the lab both before and after these experiments were performed, (in the context of deviation of freshly isolated CD4+ T cells), resulting in restoration of proliferation[131]. Whilst the efficacy of this reagent was not formally proven and therefore remains a caveat in the interpretation of these experiments, this appears an unlikely explanation for the findings. The cytokine profiles of the cell lines were reviewed and these demonstrated robust IL-10 production in the drug-deviated cell lines (Figure 3.4).

In conclusion, the experiments in this chapter show that Th2 cells can be deviated towards a regulatory T cell phenotype in the presence of calcitriol and dexamethasone, and this requires the additional presence of IL-10 in culture. The cell lines expressed high levels of IL-10 as assessed by flow cytometry, but this is not the primary mechanism for the suppression. Further work described in the subsequent text describes the investigation of mechanisms that may contribute to regulatory T cell function.

Figure 3.10 The regulatory effect of the deviated cell lines was not reversed by TGFβ blockade or the use of anti IL-10 receptor antibody.



Top panel Cultures were performed at varying ratios of deviated (Treg) or effector (Teff) cells to responder CD4 cells (Tresp)n the presence or absence of αTGFβ and αIL-10 receptor antibodies. Following 72hrs of stimulation, proliferation was assessed by intensity of CFSE signal as measured by flow cytometry.. Figure demonstrates experiment using one individual donor.

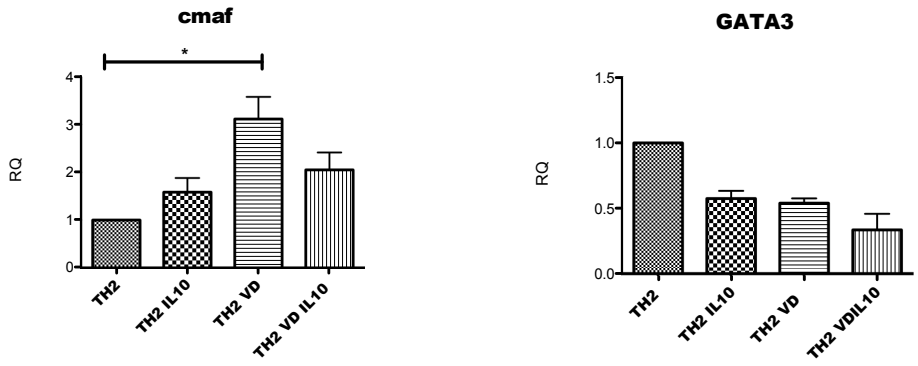
Bottom figure. Mean proliferation of autologous CD4 (Tresp) cells by CFSE staining under different culture conditions, n= 3, 3 different donors, *p<0.05, one way ANOVA.

3.2.7 Addition of calcitriol and dexamethasone to Th2 cells maintains and increases expression of c-maf, but reduces GATA3 expression

Human CD45RA+ cells were deviated towards a T_H2 profile without or with IL-10 as previously described. Cultures were restimulated weekly without (T_H2) or with addition of calcitriol plus dexamethasone (both 10^{-7} M) at day 14. mRNA was extracted as described above. Initial experiments investigated changes in the mRNA expression of the Th2-associated transcription factors c-maf and GATA3, assessed using real time RT-PCR.

Analysis of the RT-PCR data showed that an increase in c-maf was seen in the calcitriol/dexamethasone/IL-10 treated cells despite the deviation towards an IL-10 Treg cytokine profile (Figure 3.11). The increase in c-maf was sustained and persisted until day 28 of culture. In contrast, GATA3 expression was reduced in the calcitriol/dexamethasone/IL-10 cells, decreasing progressively with time while expression of GATA3 was maintained in the T_H2 cells (Figure 3.11 and data not shown).

Figure 3.11 Analysis of expression of known Th2 transcription factors



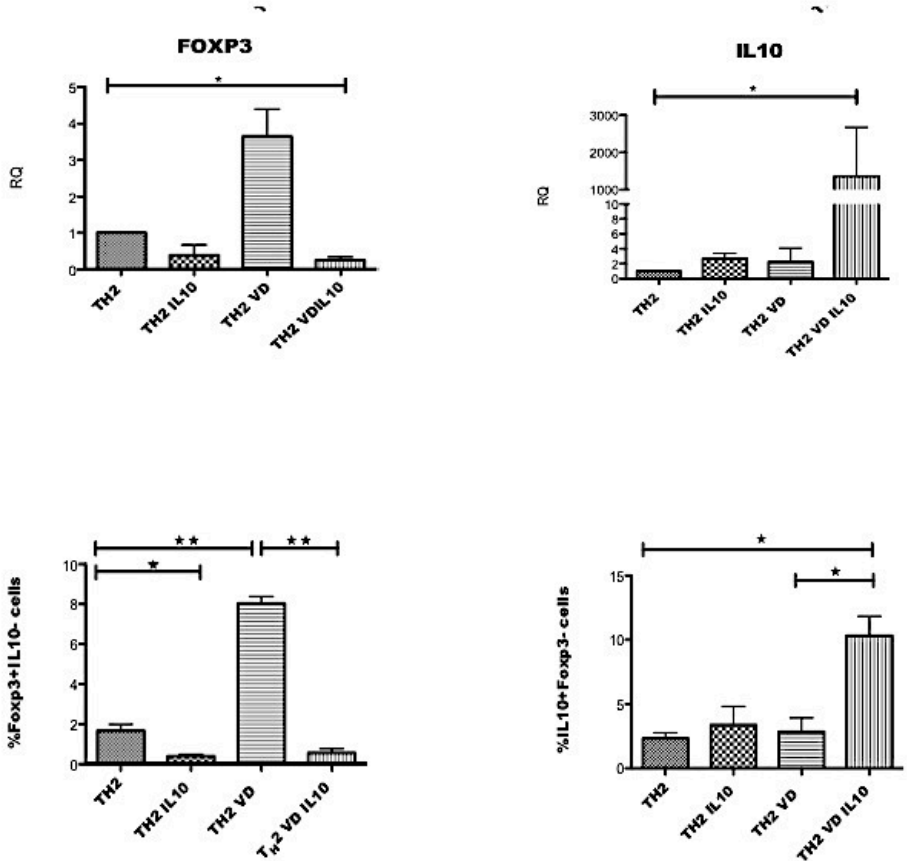
Cells were cultured under Th2 deviating conditions, some in the presence of IL-10 (Th2 IL-10) or with addition of 1α,25(OH)$_2$D3, plus dexamethasone (both at 10^{-7}M) from day 14 of culture.
Cell pellets were obtained at d28 and mRNA was extracted from the cell pellets. Quantitative rR-PCR was performed using cDNA created from the mRNA . Data represents mean + SEM of 3 experiments, 3 different donors, *p<0.05, one way ANOVA

3.2.8 Foxp3 expression is increased in Th2 cells by addition of calcitriol and dexamethasone. The increase in Foxp3 expression is abrogated by the addition of IL-10

Human CD45RA+ cells were deviated towards a Th2 profile without or with IL-10 as previously described. Cultures were restimulated weekly without (T_H2) or with addition of calcitriol plus dexamethasone (both 10^{-7} M) at day 14. The mRNA expression of Foxp3 was analysed and all conditions were compared to Th2 at day 7. Cells treated with calcitriol and dexamethasone alone showed an increase in Foxp3 expression in comparison to the Th2 cells. The expression was abrogated by the addition of IL-10 to culture (Figure 3.12). There was a reciprocal pattern of IL-10 versus Foxp3 mRNA expression, with the drug plus IL-10 treated Th2 cells showing significantly higher IL-10 gene expression than the control Th2 line. Th2 cells cultured with IL-10 or Th2 cells treated with drugs (but no IL-10) were not significantly different in IL-10 mRNA expression from the control Th2 cell lines.

Analyses of these patterns by flow cytometry demonstrated a similar pattern of protein expression with elevated levels of Foxp3 seen with the calcitriol and dexamethasone treated cells vs the Th2 VD IL-10 cells treated with exogenous IL-10 (Figure 3.13).

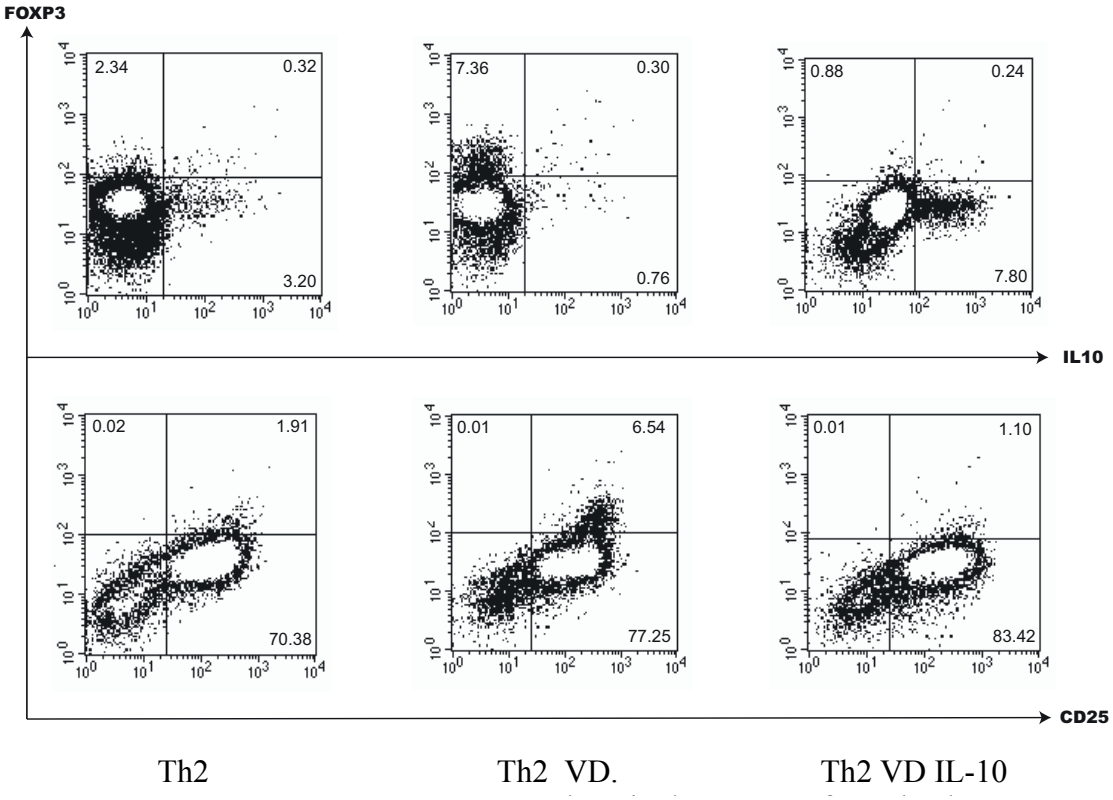
Figure 3.12 Analysis of mRNA and protein expression of Foxp3 and IL-10



Top figure. Cells were cultured under Th2 deviating conditions, some in the presence of IL-10 (Th2 IL-10) or with addition of 1α,25[OH]D3 plus dexamethasone (both at 10^{-7}M) from day 14 of culture.
Cell pellets were obtained at d27 and mRNA was extracted from the cell pellets. Quantitative RT-PCR was perfumed using cDNA created from the mRNA. Data represents mean + SEM of 3 experiments, one way ANOVA, * $p<0.05$

Bottom figure. Cells were cultured under Th2 deviating conditions, some in the presence of IL-10 (Th2 IL-10) or with addition of 1α,25 [OH] D3 plus dexamethasone (both at 10^{-7}M) from day 14 of culture.
An IL-10 secretion assay was performed overnight on cells obtained at d28 of culture. Intracellular staining was then performed to assess expression of IL-10 and Foxp3. Data represents mean + SEM of 3 experiments, one way ANOVA, * $p=<0.05$, ** $p=<0.01$

Figure 3.13 Flow cytometric analysis of Foxp3, IL-10 and CD25 expression.



Cells were cultured under Th2 deviating conditions, some in the presence of IL-10 (Th2 IL-10) or with addition of 1α,25 [OH] D3 plus dexamethasone (both at 10^{-7} M) from day 14 of culture.

An IL-10 secretion assay was performed overnight on cells obtained at d28 of culture. Intracellular staining was then performed to assess expression of IL-10 and Foxp3. Data representative of 3 experiments.

3.3 Discussion

The initial experiments described in this chapter confirmed that human Th2 cell lines can be maintained *in vitro* after several rounds of differentiation without the use of a blocking IL-10 antibody. The cytokine profile of these cell lines favoured a Th2 rather than a Th0 phenotype with high levels of IL-13 and IL-5, and low levels of IFN gamma. IL-10 production was seen initially but was transient. The human Th2 cell lines were relatively robust with the repetitive stimulus and had a singular Th2 profile without co-expression of Th17 as seen by other authors[145].

The experiments presented here also demonstrate that calcitriol and dexamethasone in combination with IL-10, when added to the Th2 cell line cultures, significantly increases the frequency of IL-10+ T cells and reduces the IL-13+ T cells, indicative of a more regulatory profile. The capacity for deviation exists even late into the Th2 cell differentiation process, occurring as late as the addition of drugs 21 days into culture.

Calcitriol and dexamethasone abrogated production of the Th2 cytokines when added to the culture but did not stimulate IL-10 production, which required the addition of exogenous IL-10. This may reflect an endogenous requirement for APCs to produce IL-10 *in vivo* to support skewing towards an IL-10 synthesising T cell population[35], particularly when calcitriol and dexamethasone are used in a milieu of Th2 effector cytokines.

Having established that the drugs could deviate the cell lines, it was important to investigate whether this represented true deviation rather than outgrowth of a single population. TCR Vβ usage in the Th2 cell line appeared polyclonal as would be expected from the mode of stimulation and the pattern did not alter upon deviation of the Th2 cells using calcitriol, dexamethasone, and IL-10, suggesting the observed results did not represent outgrowth of a minor T cell population. However, improved technologies such as newer cell proliferation dyes that can be used for a much longer period in culture (e.g. CellTraceViolet

that the lab has used for 2-weeks or longer in culture) would allow additional investigation of this issue, for example by combining CellTraceViolet with IL-13 and IL-10 staining in a time course study.

The generation of IL-10 and reduction of Th2 cytokines signifies a change in phenotype but the capacity to regulate and suppress effector responses required confirmation in a further set of functional experiments. Cells that were deviated in the polyclonal culture demonstrated suppression of autologous total CD4s in co-culture, supporting the hypothesis that culture of the Th2 cells with the immunosuppressive drugs and cytokine IL-10 leads to a regulatory phenotype. This suppression appeared potent as even at a ratio of 1Treg (deviated Th2 cell) to 16 effector cells there was no recovery of the Tresponder proliferative response. This suppression was not reversed in the presence of anti-TGF-ß antibody, or albeit in only a single experiment, by a CTLA4 inhibitory antibody. Surprisingly IL-10 blocking antibody also did not reverse this effect, as might have been expected given the increased frequency of IL-10+ cells in the deviated population, and if the regulatory effect was mediated by the suppressive cytokine alone. This result was unexpected and an important line to pursue would be whether combinations of the blocking antibodies used would lead to some recovery of effector T cell proliferation. However given that no effect of any of these antibodies was observed it is debatable whether this would be the case.

Preliminary investigation of the deviated cells exhibiting suppressive function (Th2 VD IL-10) suggested that these cells did not show increased expression of Foxp3 and therefore the drugs plus IL-10 were not causing augmentation of natural Treg responses. Increased Foxp3 expression was observed in Th2 cells that had been treated with drugs, but no IL-10, however this cell line did not exhibit suppressive activity in the *in vitro* suppression assay used. It therefore seems more likely that the increased Foxp3 expression may be more analogous to activation-dependent Foxp3 expression as previously reported to occur in human T cells[33]. Thus, in the experimental system used

here there was reciprocal expression of Foxp3 expression and IL-10 production, and exogenous IL-10 appeared to prevent Foxp3 expression.

Our lab previously reported a similar pattern of responsiveness in cultures of freshly isolated CD4+ T cells stimulated in the presence of calcitriol. Whether IL-10 or Foxp3 was preferentially increased in culture was influenced by both calcitriol concentration and the cytokine milieu; here also IL-10 had a negative effect on Foxp3 expression. However in that system both the IL-10 and Foxp3+ T cell populations exhibited comparable suppressive function[144] although the reason and significance of this is unknown.

In the Th2 VD IL-10 deviated cell lines, GATA3 mRNA expression was reduced but c-maf expression was maintained and even increased, and this may reflect the influence of c-maf on the IL-10 promoter [146]. Blockade of the AhR (Aryl hydrocarbon receptor) was not tested to see if this affected the increase in expression of IL-10 seen in concordance with the increase in cmaf expression[147].

Whilst the Th2 cell line was robust, it remains an artificial experimental *in vitro* system and it would have been advantageous to achieve a more physiological design to the experiments to test the deviation hypothesis. Experiments were performed on cells isolated *ex vivo* to try and achieve a more physiological replication of Th2 conditions and results demonstrated the complexity of using such an approach. IL-13+ cell numbers after use of the secretion assay were low after repeated rounds of stimulation which may reflect the use of stimulation with anti-CD3 and IL-2 rather than using anti-CD3 and anti-CD28 assuming that the effector cells were robust in phenotype. Alternatively a decrease in IL-13+ve cells may favour the theory of potential plasticity of the Th2 phenotype[21]. It may have been advisable to use a marker other than the cytokine IL-13 to identify Th2 cells. Other authors have suggested the use of CRTH2 as a marker for Th2 cells[148], but this marker is not specific to Th2 cells. Combinations of antibodies to detect CRTH2, and certain chemokine receptors (e.g. CCR4) and/or the IL-25 receptor may represent better

strategies to improve *ex vivo* detection of Th2 cells in humans in the future. New technologies such as single cell PCR might also enable this type of future work where very low cell numbers are available.

Further work leading on from these results would aim to shed further light on the robustness and plasticity of the cell lines and the deviated cells, further clarification of their phenotype, as well as the mechanism of suppression.

Investigation of stability and plasticity of the Th2 effector cell lines generated *in vitro* would involve withdrawal of the Th2 cytokine stimuli for longer rounds of stimulation (as opposed to a brief withdrawal used in the co-culture experiments with CFSE at d28). The cell lines would also be tested for ability to maintain the Th2 phenotype in other effector conditions by culture in Th1 or Th17 conditions.

Further investigation of the deviated, IL-10+ cell phenotype could be done using a cell surface approach to delineate further cell surface markers or alternatively a molecular approach to define factors leading to an IL-10 Treg phenotype. The second strategy was utilised in the following experimental chapters.

Chapter 4

Characterising the phenotype of Th2 cells deviated towards a regulatory phenotype by calcitriol, dexamethasone and IL-10

4.1 Introduction

Experiments performed and summarised in the previous chapter described the deviation of effector Th2 cell lines towards an IL-10-positive regulatory phenotype. An unexpected finding of the deviation experiments was that the regulatory capacity of the cells was not abrogated by the addition of anti IL-10 receptor antibody to culture, or by the addition of a neutralising antibody specific for TGF-β. This result inferred that there are likely to be additional mechanisms involved in the suppressive activity of the drug treated cells that were not related to the actions of IL-10 or TGF-β. It is uncertain if CTLA4 contributes to the suppression observed given the single experiment performed using an anti-CTLA4 neutralising antibody, where blocking again did not restore effector T cell proliferation, and this would need further experiments to clarify. These findings may therefore reflect the broader repertoire of regulatory T cell action in relation to suppression of effector cell function[23]. The cells deviated in the presence of vitamin D and dexamethasone alone in the absence of IL-10 were found to have increased expression of the transcription factor Foxp3, the most common marker of Treg. Surprisingly, these cells were not inhibitory in a conventional suppression assay. Foxp3 expression in this instance is likely to represent T cell activation[33] rather than a regulatory phenotype,

Preliminary investigation of the phenotype of the regulatory cell lines focused on the assessment of transcription factors that have historically been associated with T helper cell development. Conventionally the Th1 phenotype is promoted by T-bet expression, and GATA-3 expression favours Th2 lineage expression with contribution by c-maf. Interestingly, c-maf is also linked to IL-10 expression[146]. The main transcription factor expressed in Th17 cells is RORyt (RORC in humans)[4]. Sakaguchi identified Foxp3 as the pivotal transcription factor required for development of natural Treg[149]; conversely the level of expression of this transcription factor in induced Treg populations, such as IL-10 Treg, can be low or undetectable[131]. It is now clear that T cell

phenotypes exhibit plasticity dependent on the differential expression and interaction of transcription factors.

The lab has previously taken two experimental approaches to investigate transcriptional factors and antigens that might be specifically expressed by drug induced Treg populations, and which might therefore provide an indication as to the developmental origins of these cells, and/or their function. In the first approach the scientific literature at the time was reviewed to identify likely candidates. This approach lead to the identification of toll like receptor (TLR9) as one of the few, if only, genes/molecules to be specifically highly expressed by drug-treated CD4+ T cells that had been selected by live cytokine secretion assay for IL-10-positivity. Notably, and unexpectedly, ligation of this receptor by specific agonist (ODN-CpG) on the calcitriol /dex induced IL-10+ Treg lead to decreased IL-10 synthesis and increased IL-4 levels. The second approach used conventional gene transcriptional arrays, performed by Dr Patricia Ozegbe when she was a postdoctoral fellow in the lab, on highly differentiated drug-treated CD4+ T cells, again selected for IL-10-positivity and compared against drug treated IL-10 negative T cells, and cells activated in the absence of drugs.

Hypothesis

Effector Th2 cell lines deviated towards a regulatory phenotype with 1α,25 $[OH]_2D3$, dexamethasone and IL-10 have a specific molecular signature that defines them within the subset of induced regulatory T cells. This signature can be defined at the RNA and protein level.

Experimental aims

To identify genes of interest that represent biomarkers of, or may influence the regulatory phenotype, of Th2 cells deviated towards an IL-10+ Treg phenotype following addition of calcitriol (vit), dexamethasone (dex) and IL-10 to the Th2 culture, by:

- Screening by qRT-PCR for the expression of genes of interest identified by earlier gene transcriptional arrays performed in our lab on drug-treated IL-10+ CD4+ T cells (as opposed to Th2 cell lines) using microarray technology, and by candidates identified in the scientific literature e.g. TLR22 and TLR99.

- Perform transcriptional gene array analyses on IL-10+ 'reg generated following deviation of Th2 cell lines by the addition of calcitriol, dexamethasone and IL-10 to culture, using the methodology optimised in the previous chapter.

- Validate the microarray analysis by confirming the mRNA expression of genes of interest.

- If pathways identified, explore the effect of blocking the pathways on the regulatory phenotype.

4.2 Results

4.2.1 Genes regulating innate immunity are not upregulated in deviated regulatory Th2 cells cultured with 1α,25 $[OH]_2$D3, dexamethasone and IL-10

A microarray that had previously been performed by the Hawrylowicz lab (Dr Patricia Ozegbe) on total (naïve and memory) peripheral blood derived CD4 T cells treated with 1α,25$[OH]_2$D3 and dexamethasone (without prior culture in Th2 deviation conditions) was initially used to identify potential genes of interest in the Th2 deviated cell population. Cells cultured in this experimental protocol[131] demonstrated upregulation of a number of genes in the IL-10 positive cells in comparison to the IL-10 negative population (Ozegbe & Hawrylowicz, unpublished). In addition expression of certain TLR by the same cells was identified in a separate study by Dr Zoe Urry, following a review of the relevant scientific literature[39]. These upregulated genes were primarily associated with the innate immune response (Table 1) and this preliminary list suggested avenues for further investigation of the regulatory T cell phenotype. Upregulated genes included the toll like receptors TLR2 and TLR9, an effect strongly linked to the effects of vitamin D although ligation of these receptors may decrease rather than increase IL-10 production[39].

Table 3 Upregulated genes Ozegbe microarray

	Function
SERPINA1	Serine Protease inhibitor[150], encoding for the protein alpha1 antitrypsin
TENASCIN C	Extracellular matrix glycoprotein[151]
TIMP 2	Tissue inhibitor of metalloproteinase[152]
CLEC 7A	Non-toll-like pattern recognition receptor[153]
TLR 2	Toll like pattern recognition receptor[154]
TLR 9	Toll like pattern recognition receptor[39]
IGF1	Insulin like growth factor 1[155]

4.2.2. Analysis of TLR2, TLR9 and SERPINA1 mRNA expression

Earlier work in our lab demonstrated upregulation of TLR9 in drug induced IL-10+ Treg, which was one of the few genes associated with an IL-10+ phenotype[39]. There was also upregulation of TLR-2, which has been associated with regulatory responses[154]. SERPINA, which codes for the protein alpha 1 antitrypsin, a serine protease inhibitor, was also upregulated - notably, there is evidence that inhibition of serine protease activity may be involved in Th2 responses[156]; and alpha 1 antitrypsin has been reported to increase IL-10 synthesis by monocytes[157].

Experiments to analyse the phenotype of the deviated Th2 cells assessed the mRNA expression of the above genes as shown in table 4.1 in the effector (Th2) and deviated (Th2VDIL-10) cell lines. Th2 cell lines were cultured for a total of 28 days with 1α,25 $[OH]_2$D3 and dexamethasone added at either day 7 or day 14 of culture, with or without IL-10 added at day 0. Total RNA was

isolated from cell pellets at various time points indicated and reverse-transcribed to create cDNA. mRNA expression was determined by PCR amplification and quantification.

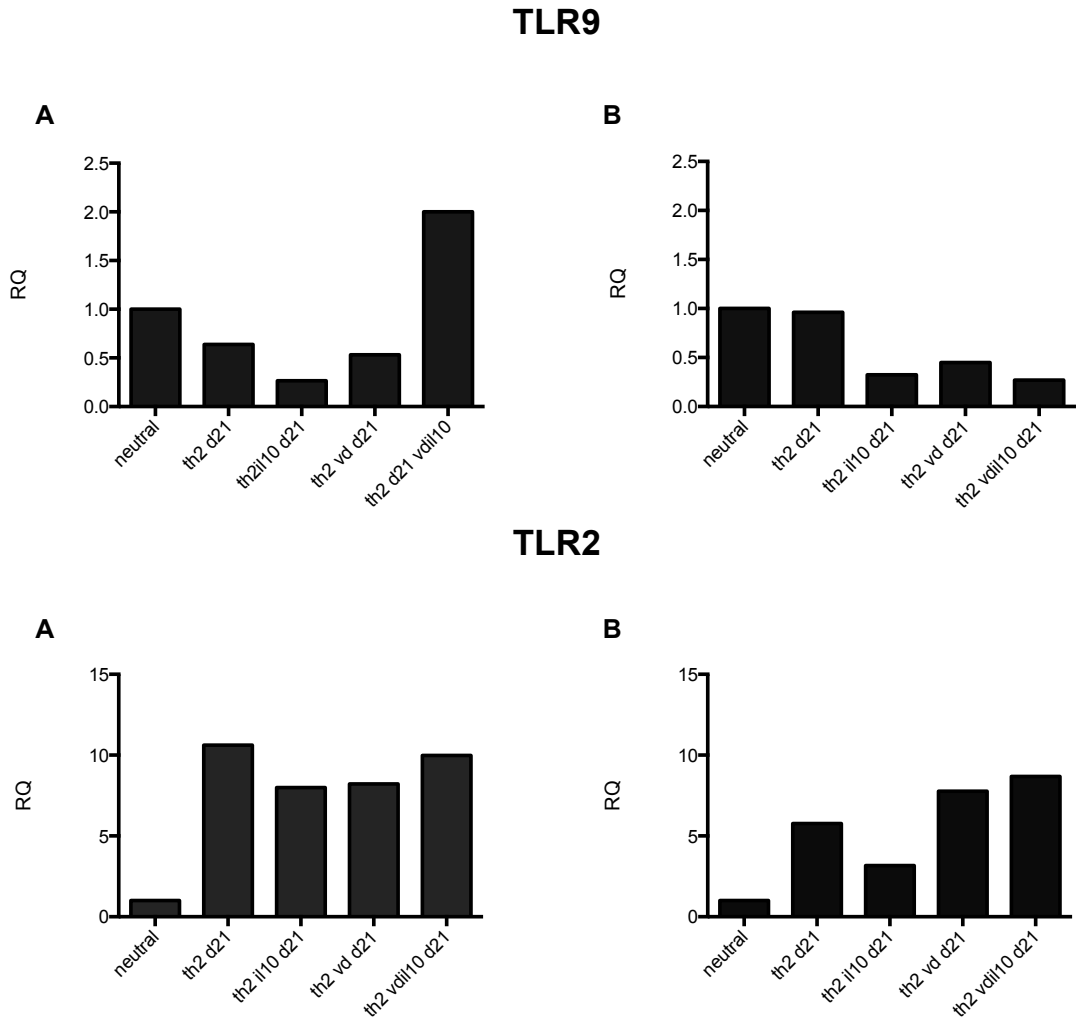
TLR9 mRNA expression did not change significantly in cells treated with the 1α,25 $[OH]_2$D3 and dexamethasone alone by day 14 (after 7 days of culture with drugs). By day 21 there was a modest increase in TLR9 expression (Figure 1) in one of the two donors studied, and observed only with the full combination of 1α,25 $[OH]_2$D3, dexamethasone and IL-10.

TLR2 expression was increased in cells cultured in the Th2 cell lines (Figure 4.1) in both donors tested. However there was no convincing change in expression with the addition of IL-10, 1α,25 [OH]D3 and dexamethasone, or 1α,25 $[OH]_2$D3 and dexamethasone plus IL-10.

Analysis of expression of the SERPINA1 gene in the deviated Th2 cell lines showed consistent results between the two donors. In comparison to the T cell lineage generated under Dr Patricia Ozegbe, there was downregulation and decreased mRNA expression in the deviated cell line cultured with 1,25 $[OH]_2$D3, dexamethasone and IL-10 (Figure 4.2).

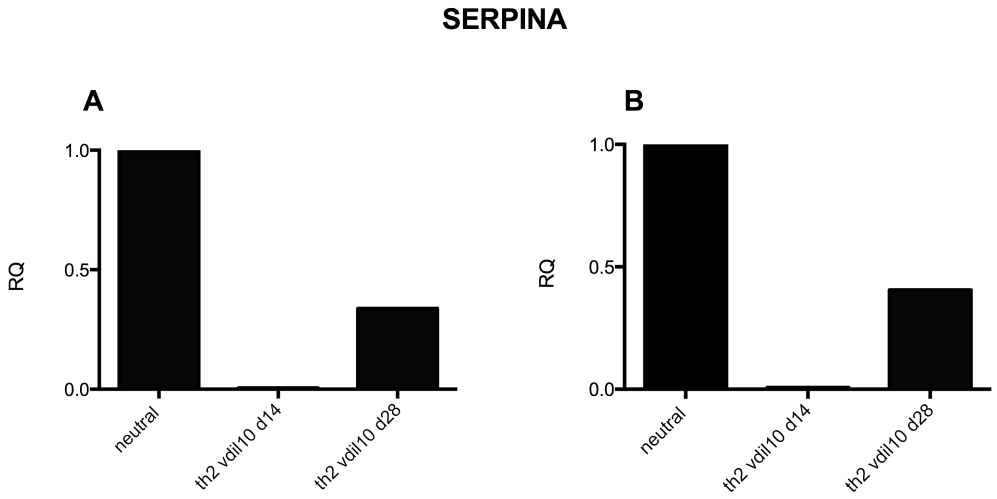
Gene expression of IGF1 increased under all culture conditions with time, and was highest in cells treated with 1α,25 [OH]D3 and dexamethasone, with the highest levels of expression seen with the addition of IL-10 (Figure 4.3) in both donors. This increase in IGF1 was not explored further as analysis was superseded by the new microarray (see below). It may reflect an increase in genes related to tissue repair, as demonstrated by the increase in tenascin expression seen in the Ozegbe array.

Figure 4.1 Analysis of TLR9 and TLR2 expression – representative PCR experiments



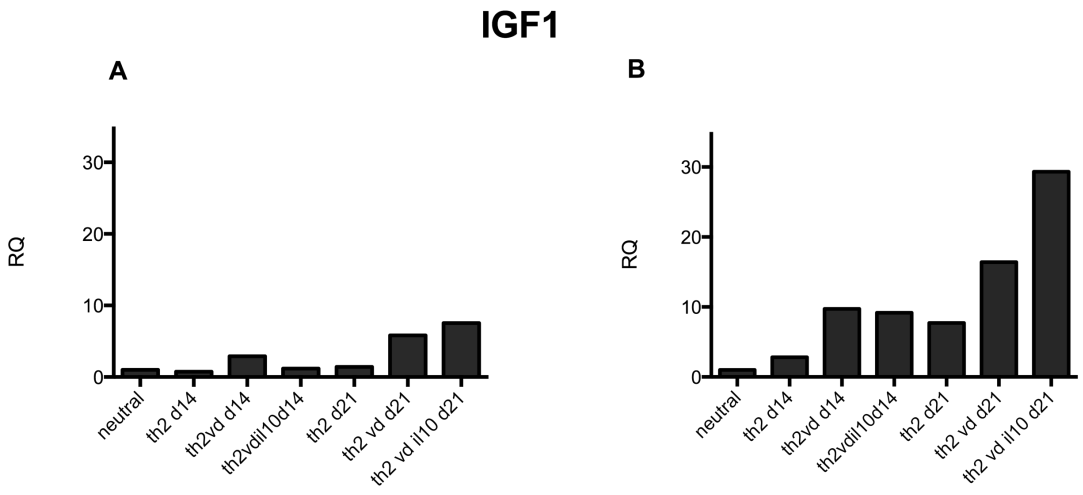
Cells were cultured under Th2 deviating conditions (th2), some in the presence of IL-10 (IL-10), or with addition of 1α,25 [OH]D3 (v) and dexamethasone (d; both at 10^{-7}M) from day 7 of culture Cell pellets were obtained at d21 and mRNA was extracted for analysis. Quantitative RT–PCR was performed using cDNA created from the mRNA. Th2 cell lines or deviated cells were compared to pellets from d14 neutral cells grown in the presence of IL-2 only and restimulated at d7 with anti-CD3. A donor 1, B donor 2

Figure 4.2 Analysis of SERPINA expression



CD4 cells were cultured under Th2 deviating conditions, in the presence of IL-10 (at different concentrations) and addition of 1α,25 $[OH]_2D3$ and dexamethasone (both at 10^{-7}M) from day 7 of culture. Cell pellets were obtained at d28 and mRNA was extracted from the cell pellets. Quantitative rT –PCT was performed using cDNA created from the mRNA. Th2 or deviated cells were compared to pellets from d14 neutral cells grown in the presence of IL-2 only and restimulated at d7 with anti-CD3.
A donor 1, B donor 2.

Figure 4.3 Analysis of IGF1 expression



Cells were cultured under Th2 deviating conditions, in the presence of IL-10 with addition of 1α,25 $[OH]_2D3$ and dexamethasone (both at 10^{-7}M) from day 7 of culture. MRNA was extracted at day 21 and PCR performed on cDNA created from the mRNA. A and B, two individual donors.

4.2.3 Utilisation of a new microarray strategy to investigate the deviated cell phenotype

On review of the results relating to expression of genes previously identified to be upregulated genes in the original array of calcitriol plus dexamethasone induced IL-10+ Treg, the majority of experiments, with the exception of IGF1, failed to demonstrate a similar gene profile in the Th2 deviated regulatory cell lines and this approach was discontinued. This finding stimulated further work on the deviated Th2 cell lines to identify a molecular signature that would provide a biomarker(s) and /or account for their regulatory action. A new microarray strategy was developed and initiated and the results were analysed to identify genes with the highest expression in the regulatory cells, as well as the highest level of fold change between the Th2 cell lines and the various drug treated conditions. Results for genes of interest were then validated by analysis of mRNA expression.

4.2.4 Protocol for selection of cell lines and pooling of cell lines for microarray

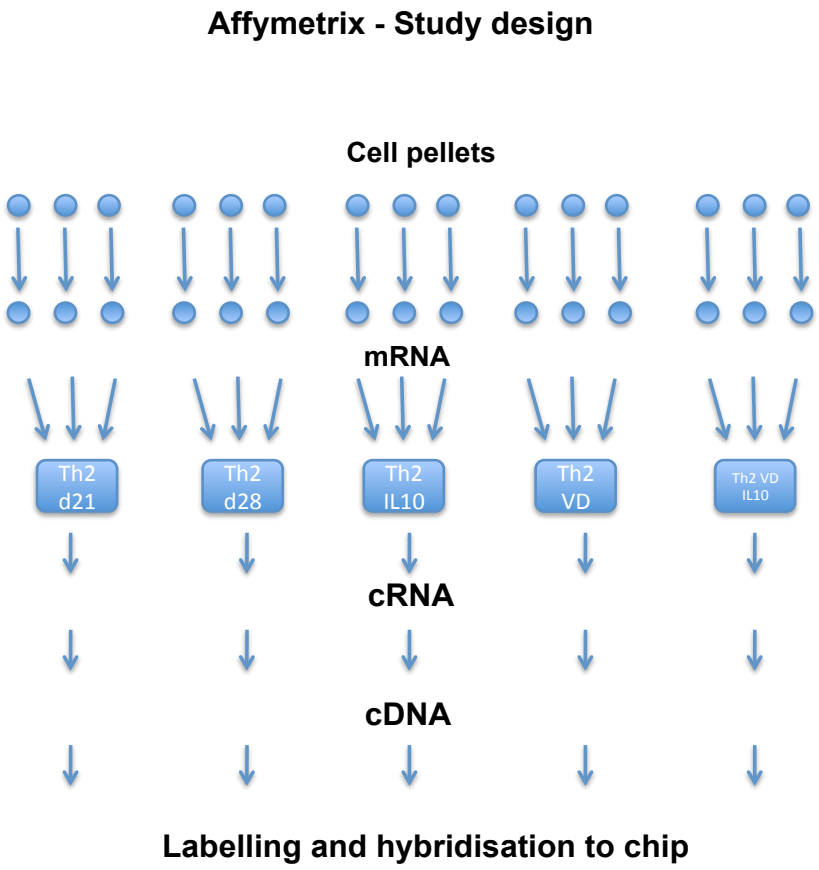
Th2 cell lines were cultured in deviating conditions as described in the previous chapter, some in the presence of IL-10 (Th2 IL-10) with or without addition of 1α,25 [OH] D3 plus dexamethasone (both at 10^{-7} M) from day 14 of culture.

mRNA was extracted from cell pellets obtained from 3 individual cell lines from each of the conditions for the effector Th2 line and the deviated lines resulting in four different cells lines: (i) Th2;(ii) Th2+IL-10; (iii)Th2 with the addition on day 14 of culture of 1α,25 $[OH]_2$ D3 plus dexamethasone and (iv).

Th2+IL-10 with the addition on day 14 of culture of 1,25 [OH] D3 and dexamethasone. The mRNA samples were pooled for each condition and cDNA was transcribed using the Ambion WT expression kit (as per protocol). Samples were pooled rather than used individually due to constraints in availability of the array chip, but it was felt that the data would still be representative, and the aim was to then validate the data of interest by q RT-PCR in remaining material from the four cell lines derived from each of the three donors individually. The limitations of this experimental approach are addressed further both in the Results section and in the Discussion of this chapter. A fifth condition was also added (Th2 d21) in order to facilitate normalisation of the array result. cDNA was labelled and hybridised to the Exon ST array chip (Affymetrix) as per the manufacturer's protocol.

Protocol for selection of cell lines and pooling of cell lines for microarray

Figure 4.4 Study protocol for selection of samples for Exon ST microarray analysis



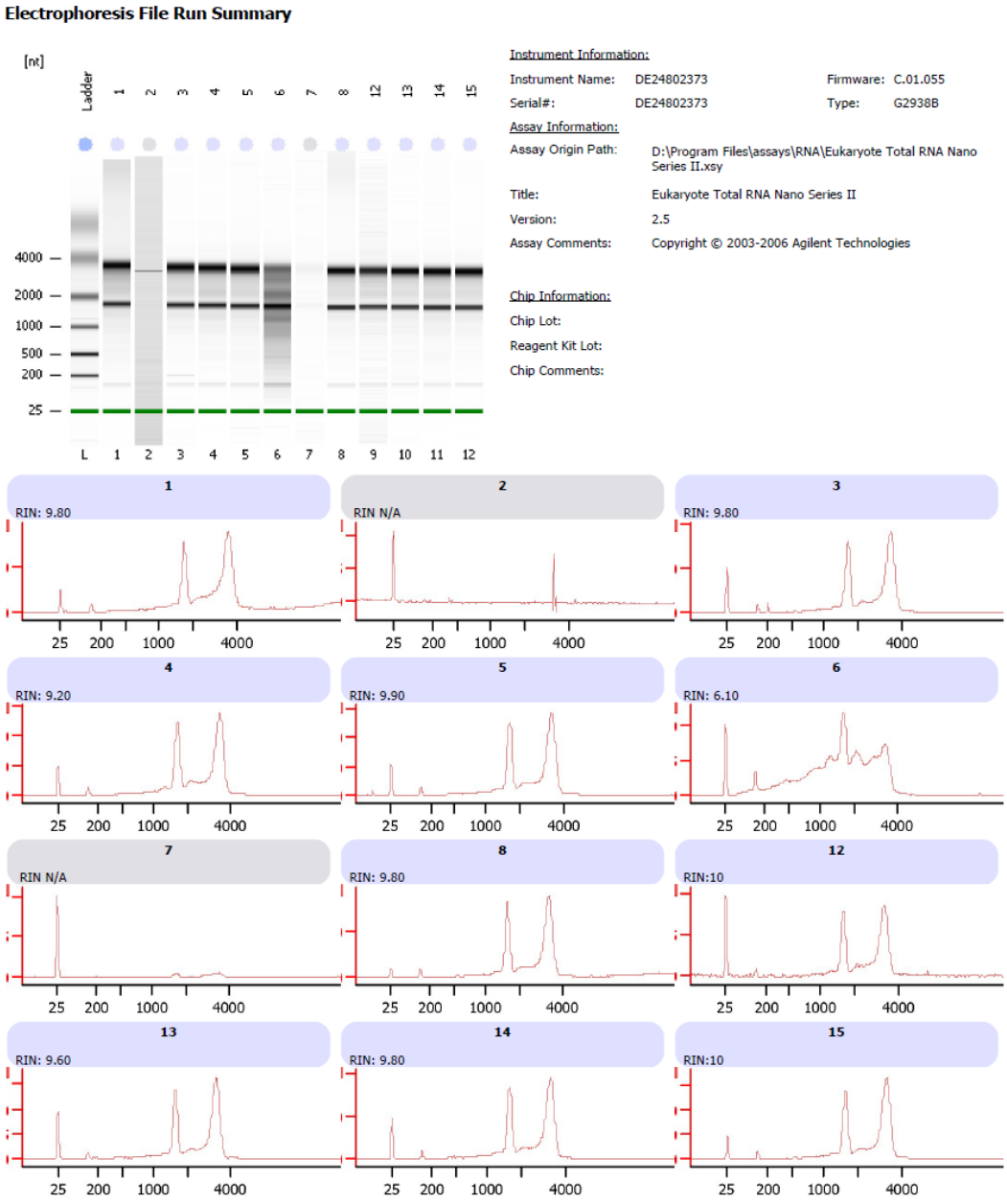
Cells were cultured under Th2 deviating conditions, some in the presence of IL-10 (Th2 IL-10) or with addition of 1,25 [OH] D3 plus dexamethasone (both at 10^{-7} M) from day 14 of culture.

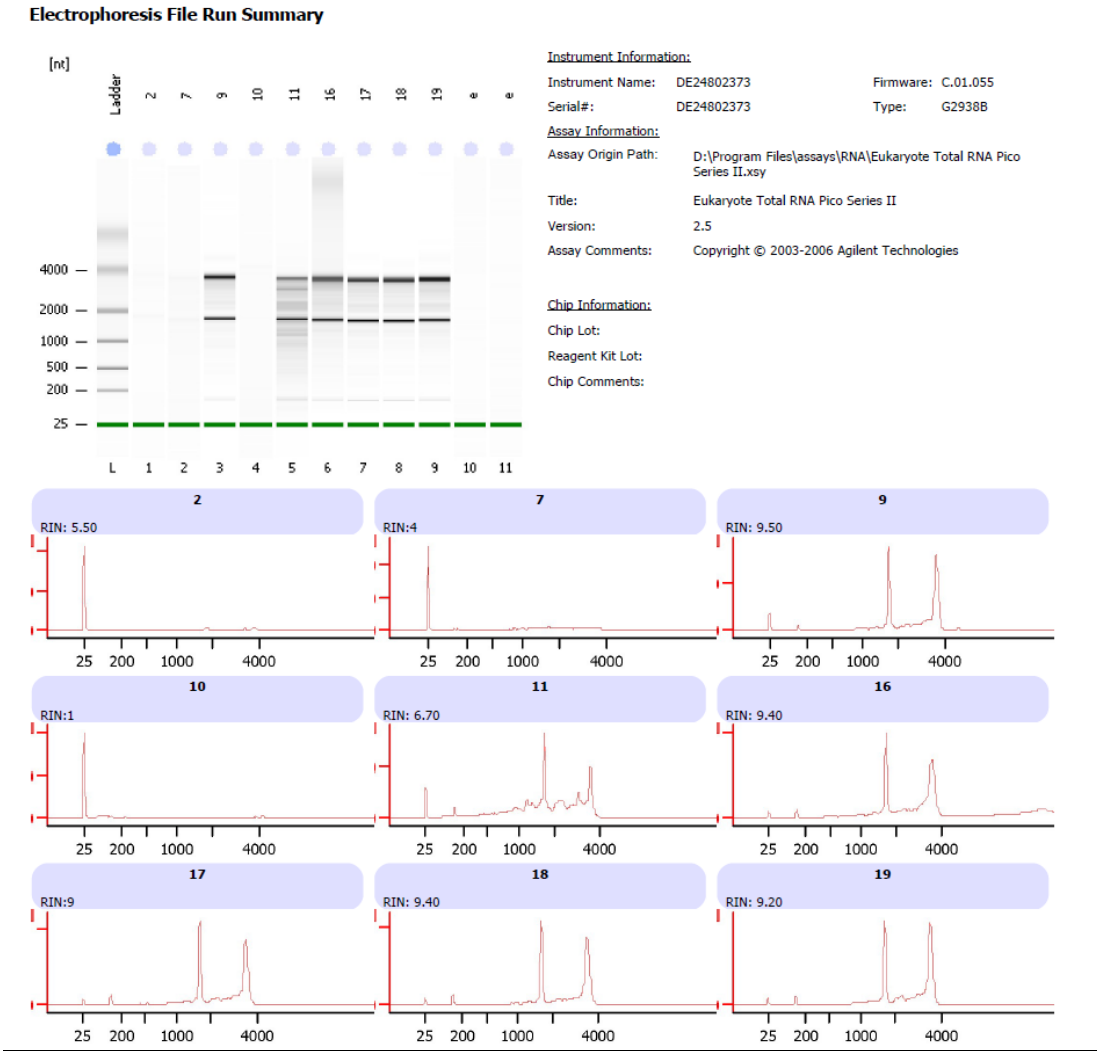
mRNA was extracted from cell pellets obtained from 3 individual cell lines for each of the conditions outlined above. The mRNA samples were pooled for each condition and cDNA was transcribed using the Ambion WT expression kit (as per protocol). A control condition was also added (Th2 d21) to ensure the validity of the array analysis. cDNA was labelled and hybridised to the Exon ST array chip (Affymetrix) as per protocol.

4.2.5 mRNA quality control analysis

mRNA samples from cell pellets derived from the individual experiments were examined to determine mRNA integrity prior to pooling using the bioanalyzer system. Sample details are listed below.

Figure 4.5 mRNA quality control analysis





Cells were cultured under Th2 deviating conditions, some in the presence of IL-10 (Th2 IL-10) or with addition of 1,25 [OH] D3 plus dexamethasone (both at 10^{-7} M) from day 14 of culture.

mRNA was extracted from cell pellets obtained from 3 individual cell lines for each of the conditions outlined above. The mRNA samples were assessed using a bioanalyser. See table 4 for description of the samples.

Table 4 mRNA samples for exon ST microarray

Sample no	Experiment no	Condition	Day of culture prior to isolation
1	34	Th2	21
2	70	Th2 VDIL-10	28
3	34	Th2 IL-10	28
4	34	Th2 IL-10	28
5	34	Th2 VD	28
6	34	Th2	28
7	70	Th2	28
8	70	Th2	21
9	70	Th2 VD	28
10	70	Th2 IL-10	28
11	69	Th2 IL-10	28
12	37	Th2 VD	28
13	37	Th2 IL-10	28
14	37	Th2 VDIL-10	28
15	37	Th2	28
16	69	Th2	21
17	69	Th2 VD	28
18	69	Th2	28
19	69	Th2 VDIL-10	28

4.2.6 Exon ST microarray analysis

The Exon ST microarray of the four cell lines under study was run within the Biomedical Research Centre Genomics facility. The microarray results were reviewed initially to determine the genes with the highest intensity following normalisation. Due to sample constraints (and cost) the samples for each condition had to be pooled (as per experimental protocol outlined in Figure 4.4) rather than used individually. This precluded detailed biocomputational analysis to enable determination of genes with statistically significant upregulation or downregulation. A pragmatic approach was taken with regard to the analysis on this basis. The gene lists were compared to identify the greatest fold change in gene expression between the Th2 cell lines and the deviated Th2 VDIL-10 cells. The lists were then scrutinised to identify gene ontology groupings (Tables 1 and 2).

Evaluation of fold changes between the Th2 VDIL-10 cells and Th2 cell lines demonstrated that there were high levels of intensity seen for CYP24A1, an enzyme strongly associated with vitamin D metabolism (breaks down the active form of vitamin D into its metabolites)[107]. This provided important evidence that the cells had responded appropriately to the 1α,25 $[OH]_2$ D3 added to cultures as CYP24A1 is known to be highly upregulated by 1α,25$[OH]_2$D3 and thus provides a positive control for calcitriol actions in culture, as a recognised "vitamin D response gene". However, there was no change in fold expression related to cathelicidin, an important vitamin D response gene in relation to innate immunity. Both cell lines had similar mean intensity.

Legumain (LGMN) is a cysteine protease that cleaves vitamin D from its binding protein[158]. High expression of the gene for legumain was observed in the Th2VDIL-10 deviated cell line in comparison to the Th2 cell line (fold increase of 32). Legumain not only has a function in vitamin D metabolism alone, but has previously been noted to have an influence on phenotype of natural (Foxp3+) Treg cells[159].

Analysis of fold change in relation to gene ontology demonstrated a number of groupings related to biological processes. There were a number of genes expressed related to proteolysis. Legumain, as previously described, was described within the category of proteolytic genes. A number of granzyme genes were also upregulated with an increased fold change between the Th2 cell lines and the drug deviated cells (even more so than upregulation of IL-10). Granzymes are serum proteases that induce cell death and have been identified in other Treg cell subtypes[160,161].

A number of genes related to signal transduction were identified, including TAAR6 (polymorphisms of this gene may be associated with asthma), OR4N4 and OR13C5, members of the olfactory gene receptor family with homology to hormonal receptors, as well as more established molecules such as CXCR6

and CD38[144]. ELOLV4 and ELOLV6 genes were noted, which are genes that code for processing of elongated long chain fatty acids.

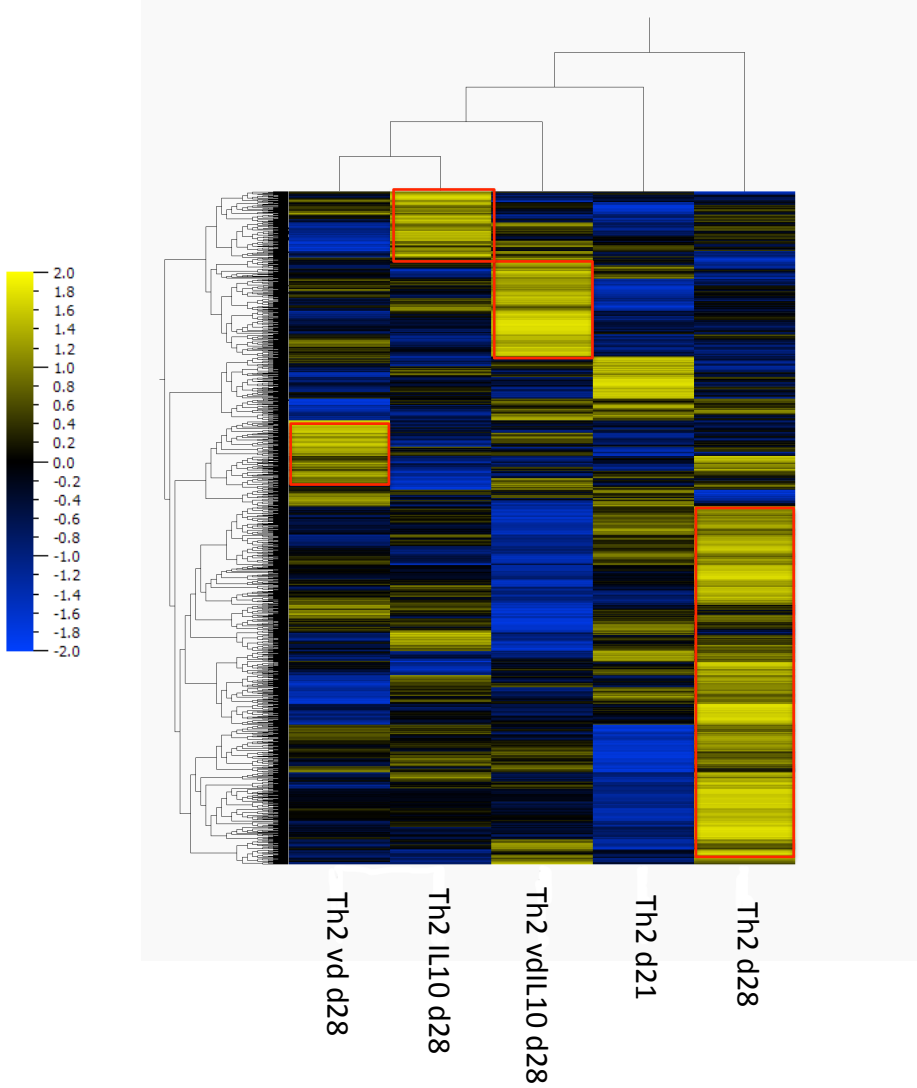
The gene ontology analysis of Th2 VDIL-10 cells also demonstrated a number of genes related to the immune system, such as BTLA 4 (B and T lymphocyte factor 4) linked to the B7:CD28 superfamily and PDCD1LG2 (programmed cell death 1 ligand 2), one of the ligands for the action of PD-1. PD-L1 has been well recognised as a marker for regulatory T cell function, and both PD-1 and PD-L1 have been reported to be increased in culture by vitamin D[144 162] The effects of PD-L2 on Treg and the lung have been variable and not as broadly characterised.

Another focal group of upregulated genes that were identified are involved in proteolysis including the granzymes (B and H) and granulysin. Upregulation of genes involved in chemotaxis (CCR1 and CCR10) and angiogenesis (VEGF and EPAS) were also noted. CCR10 has previously been reported to be increased by vitamin D[163].

On analysis of genes that were more highly expressed in Th2VDIL-10 cells as opposed to Th2 VD cells (tables 3 and 4), the granzymes again demonstrated a high profile. There was also an increase in granulysin expression noted. Granulysin was also upregulated in the Th2 VDIL-10 cells, and this enzyme is known to co-localise in granules with granzymes and perforin.

A basic heatmap of gene expression was compiled using the pooled data as shown in Figure 6 with comparable results to the analyses detailed above. Pooling of the RNA samples for the conditions precluded more detailed statistical tests on the array data to assess gene significance.

Figure 4.6 Heatmap analysis of Exon ST pooled microarray



Cells were cultured under Th2 deviating conditions, some in the presence of IL-10 (Th2 IL-10) or with addition of 1α,25 [OH] D3 plus dexamethasone (both at 10^{-7} M) from day 14 of culture.

mRNA was extracted from cell pellets obtained from 3 individual cell lines for each of the conditions outlined above. The mRNA samples were pooled for each condition and cDNA was transcribed using the Ambion WT expression kit (as per protocol). A control condition was also added (Th2 d21) to ensure the validity of the array analysis. cDNA was labelled and hybridised to the Exon ST array chip (affymetrix) as per protocol. A Heatmap was generated using bioinformatics computational analysis to identify clusters of genes based on gene expression patterns (outlined in red).

Table 5 Gene ontology analysis of ExonST microarray results for Th2 VDIL-10 cell lines vs Th2 cell lines at d28

Fold change	Gene symbol	mRNA description	GO Biological process	GO Cellular Component Term	GO Molecular Function Term
+32.8336447	**LGMN**	Legumain (LGMN), transcript variant 1	**Proteolysis**	lysosome	
+3.87984764	**GMZK**	Granzyme K (granzyme 3; tryptase II)	**Proteolysis**	extracellular region	serine-type endopeptidase activity
+5.93951446	**GMZB**	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated	**proteolysis // apoptosis // cleavage of lamin // cytolysis**	immunological synapse // nucleus // cytoso	catalytic activity // serine-type endopeptidase activity // protein binding //
+8.47121555	**GMZH**	granzyme H (cathepsin G-like 2, protein h-CCPX)	**proteolysis // proteolysis // apoptosis // cytolysis**	cytoplasm	catalytic activity // serine-type endopeptidase activity // serine-type
+3.16515028	**TAAR6**	Trace amine associated receptor 6	**signal transduction // G-protein coupled receptor**	plasma membrane // integral to membrane	rhodopsin-like receptor activity
+4.42528324	**OR4N4**	Olfactory receptor, family 4, subfamily N, member 4	**signal transduction // G-protein coupled receptor**	plasma membrane // integral to membrane	receptor activity
+3.35675211	**OR13C5**	Olfactory receptor, family 13, subfamily C, member 5	**signal transduction // G-protein coupled receptor**	plasma membrane // integral to membrane	receptor activity
+5.08721476	**CXCR6**	Chemokine (C-X-C motif) receptor 6	**signal transduction // G-protein coupled receptor**	plasma membrane // integral to membrane	C-C chemokine receptor activity
+8.33047639	**CD38**	CD38 molecule	**signal transduction // G-protein coupled receptor**	plasma membrane // integral to membrane	NAD+ nucleosidase activity

Fold change	Gene symbol	mRNA description	GO Biological process	GO Cellular Component Term	GO Molecular Function Term
+ 4.05085656	**ELOVL4**	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	**fatty acid biosynthetic process**	cellular_component // endoplasmic reticulum //	G-protein coupled photoreceptor activity
+ 8.17950195	**ELOVL6**	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like,	**fatty acid elongation**	mitochondrion // endoplasmic reticulum //	transferase activity, transferring groups other than amino-acyl groups
+3.2094494	**BTLA**	B and T lymphocyte associated (BTLA), transcript variant 1,	**immune response**	membrane // integral to membrane	receptor activity
+3.04899757	**PDCD1LG2**	Programmed cell death 1 ligand 2	**immune response**	extracellular region // plasma membrane // endomembrane system	molecular_function // receptor activity
+10.2872457	**CD79A**	CD79a molecule, immunoglobulin-associated alpha	**immune response // cell surface receptor linked signal transduction**	multivesicular body // plasma membrane // external side of plasma	transmembrane receptor activity // protein binding
+4.33665297	**PF4**	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	**immune response // negative regulation of angiogenesis /**	extracellular region //	chemokine activity // heparin binding

Fold change	Gene symbol	mRNA description	GO Biological process	GO Cellular Component Term	GO Molecular Function Term
+ 6.45867399	**VEGFA**	Vascular endothelial growth factor A (VEGFA), transcript variant A	**angiogenesis // angiogenesis // vasculogenesis // response to hypoxia**	extracellular region // extracellular region // proteinaceous extracellular	platelet-derived growth factor receptor binding // vascular endothelial
+ 7.18584093	**EPAS1**	Endothelial PAS domain protein 1 (EPAS1), mRNA	**angiogenesis // response to hypoxia // regulation of transcription, DNA-dependent**	nucleus // transcription factor complex // transcription factor complex	RNA polymerase II transcription factor activity, enhancer binding //
+ 11.5436775	**G0S2**	G0/G1switch 2 (G0S2)	**cell cycle**	cellular_component	molecular_function
+ 3.60517677	**CCNA1**	Cyclin A1 (CCNA1), transcript variant 1,	**cell cycle // mitosis**	nucleus // cytosol // microtubule cytoskeleton	protein binding
+ 7.33896186	**APBB2**	Amyloid beta (A4) precursor protein-binding, family B	**cell cycle arrest // intracellular signaling cascade // negative regulation of S phase of mitotic cell cycle**	nucleus // membrane // lamellipodium // growth cone // synapse	beta-amyloid binding // transcription factor binding
+ 3. 00403371	**CCRL1**	Chemokine (C-C motif) receptor-like 1 (CCRL1),	**chemotaxis // immune response // signal transduction // G-**	plasma membrane // integral to plasma membrane	C-C chemokine
+ 13.1074124	**CCR10**	Chemokine (C-C motif) receptor 10 (CCR10)	**chemotaxis // signal transduction // G-protein coupled recep**	plasma membrane // integral to plasma membrane	receptor activity // protein binding

Table 6 Greatest fold change between Th2 VDIL-10 and Th2 cell lines

Gene symbol	mRNA description	Fold change	Gene symbol	mRNA description	Fold change
CYP24A1	Cytochrome P450 (CYP24A1)	+44.7603062	**NEDD8**	Neural precursor cell expressed	+6.03689204
LGMN	Legumain	+32.8336447	**HPGD**	Hydroxyprostaglandin dehydrogenase 15-(NAD)	+5.99841359
NRN1	Neuritin	+21.6101623	**GZMB**	Granzyme B	+5.93951446
CCR10	Chemokine (C-C motif) receptor 10	+13.1074124	**MYO1B**	Myosin IB	+5.87731817
G0S2	G0/G1switch2	+11.5436775	**TXK**	TXK tyrosine kinase (TXK	+5.81728506
CD79A	CD79a molecule, immunoglobulin-associated alpha	+10.2872467	**IL-10**	Interleukin 10	+5.60705414
SCGB1D2	Secretoglobin,	+8.97477643	**AREG**	Amphiregulin (schwannoma-derived growth factor)	+5.28013201
SH3BGRL2	SH3 domain binding glutamic acid-rich protein like 2	+8.69388493	**KLRD1**	Killer cell lectin-like receptor subfamily D	+5.23224465
GZMH	Granzyme H	+8.47121555	**FKBP5**	FK506 binding protein 5 (FKBP5)	+5.15718572
FAM49A	family with sequence similarity 49	+8.45545271	**CXCR6**	Chemokine receptor 6	+5.08721476
CD38	CD38 molecule	+8.33047639	**P2RX5**	Purinergic receptor P2X,	+4.98926743
ELOVL6	ELOVL family member 6	+8.17950195	**LOC541472**	Uncharacterised LOC541472	+4.82116231
APBB2	Amyloid beta (A4) precursor protein-binding, family B,	+7.33896186	**DOCK5**	Dedicator of cytokinesis 5 (DOCK5)	+4.78498698
EPAS1	Endothelial PAS domain protein 1 (EPAS1)	+7.18584093	**MAP3K5**	Mitogen-activated protein kinase kinase kinase 5	+4.78498698
VEGFA	Vascular endothelial growth factor A (VEGFA)	+6.45867399	**OR4N4**	Olfactory receptor, family 4	+4.47896689
IRF8	Interferon regulatory factor 8	+6.30360878	**TSPAN2**	Tetraspanin 2 (TSPAN2)	+4.41746897

Table 7 Gene ontology analysis of Exon ST microarray results for Th2VDIL-10 cell lines vs Th2 VD cell lines at d28

Fold change	Gene symbol	mRNA description	GO Biological process	GO Cellular Component Term	GO Molecular Function Term
39.18914	**GMZB**	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated	**proteolysis // apoptosis // cleavage of lamin // cytolysis**	immunological synapse // nucleus // cytoso	catalytic activity // serine-type endopeptidase activity // protein binding //
4.81041	**GMZH**	granzyme H (cathepsin G-like 2, protein h-CCPX)	**proteolysis // proteolysis // apoptosis // cytolysis**	cytoplasm	catalytic activity // serine-type endopeptidase activity // serine-type
3.03544493	**NRIP3**	Nuclear receptor interacting protein 3 (NRIP3)	**proteolysis**	n/a	aspartic-type endopeptidase activity
5.35726254	**IL-10**	Interleukin 10 (IL-10)	**negative regulation of cytokine secretion during immune response etc.**	extracellular region // extracellular space	cytokine activity // interleukin-10 receptor binding // protein binding
3.98528242	**ELOVL4**	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	**fatty acid biosynthetic process**	cellular_component // endoplasmic reticulum //	G-protein coupled photoreceptor activity
3.537828	**CCR10**	Chemokine (C-C motif) receptor 10	**chemotaxis // signal transduction**	plasma membrane // integral to plasma membrane	protein binding // C-C chemokine receptor activity
3.1230783	**CXCL11**	Chemokine (C-X-C motif) ligand 11 (CXCL11)	**chemotaxis // inflammatory response //**	extracellular region // extracellular space	chemokine activity
3.28212685	**SLAMF7**	SLAM family member 7 (SLAMF7), mRNA.	cell adhesion // natural killer cell activation //	membrane // integral to membrane	receptor activity

Table 8 Greatest fold change between Th2 VDIL-10 and Th2 VD

Gene symbol	mRNA description	Fold change	Gene symbol	mRNA description	Fold change
GZMB	Granzyme B	+39.1891398	**RCN1**	reticulocalbin 1, EF-hand calcium binding domain (RCN1	+3.74488392
GNLY	Granulysin	+9.41409884	**NRN1**	Neuritin	+3.70788372
KLRD1	Killer cell lectin-like receptor subfamily D	+8.6082919	**ITGAX**	Integrin, alpha X	+3.70788372
FAM49A	Family with sequence similarity 49	+8.41652676	**CD9**	CD9 molecule	+3.66725097
LCE2D	Late cornified envelope 2D	+7.89884658	**CCR10**	Chemokine (C-C motif) receptor 10	+3.53782822
SH3BGRL2	SH3 domain binding glutamic acid-rich protein like 2	+6.40474441	**LOC100130492**	PREDICTED: Homo sapiens misc_RNA	+3.39061505
IL-10	Interleukin 10	+5.35726254	**PDLIM1**	PDZ and LIM domain 1 (elfin	+3.37747479
RBMS2	RNA binding motif, single stranded interacting protein 2	+5.31774648	**KLRG1**	killer cell lectin-like receptor subfamily G	+3.36223136
GMZH	granzyme H (cathepsin G-like 2, protein h-CCPX)	+4.81041046	**SLAMF7**	SLAM family member 7 (SLAMF7	+3.28212685
STOM	stomatin (STOM), transcript variant 1	+4.6994014	**CHI3L2**	chitinase 3-like 2 (CHI3L2)	+3.23872057
SCGB1D2	Secretoglobin,	+4.63276017	**AIG1**	androgen-induced 1	+3.1989335
FCRL6	Fc receptor-like 6 (FCRL6)	+4.38040278.4	**RPL23AP13**	ribosomal protein L23a pseudogene 13	+3.18414272
PLEK	pleckstrin	+4.08897431	**DAPK2**	death-associated protein kinase 2	+3.17697998
FGFBP2	fibroblast growth factor binding protein 2	+4.08222301	**CXCL11**	chemokine (C-X-C motif) ligand 11 (CXCL11	+3.13230873
ELOLV4	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	+3.98528252	**NRIP3**	nuclear receptor interacting protein 3	+3.03544493
RCN1	reticulocalbin 1, EF-hand calcium binding domain (RCN1	+3.86530163	**HAVCR2**	hepatitis A virus cellular receptor 2	+2.937712066

4.2.7 Validation of microarray by PCR analysis

4.2.7.1 qPCR analysis of mRNA expression of microarray genes involved in Treg immune responses (on gene ontology analysis) show trend of increased expression in deviated (Th2VDIL-10) cell lines

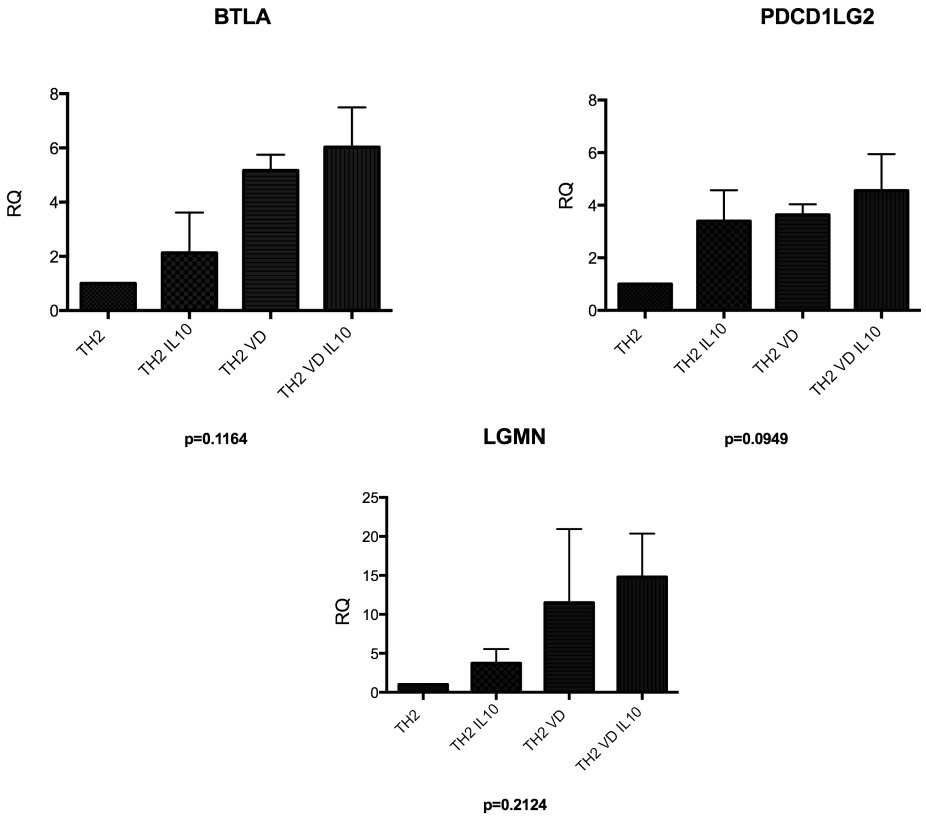
Human CD45RA+ cells were deviated towards a Th2 profile without or with IL-10 as previously described. Cultures were restimulated weekly without (Th2; Th2IL-10) or with addition of calcitriol plus dexamethasone (both 10^{-7} M) at day 14. mRNA was extracted as described above . The mRNA expression of BTLA, LGMN and PDCDLG2 in the individual mRNA samples that were ultimately pooled was assessed using real time RT-PCR.

The mRNA analyses demonstrated a trend towards increased expression of BTLA4, LGMN and PDCDLG2 in the drug treated cells compared to the Th2 cell. This was highest in the drug treated cells that were cultured in the presence of IL-10 in each of the three donors studied. However, this observation was not statistically significant, likely due to small sample size.

4.2.7.2 Granzyme expression is highly expressed in VDIL-10 cells compared to Th2 cells and Th2 VD cells

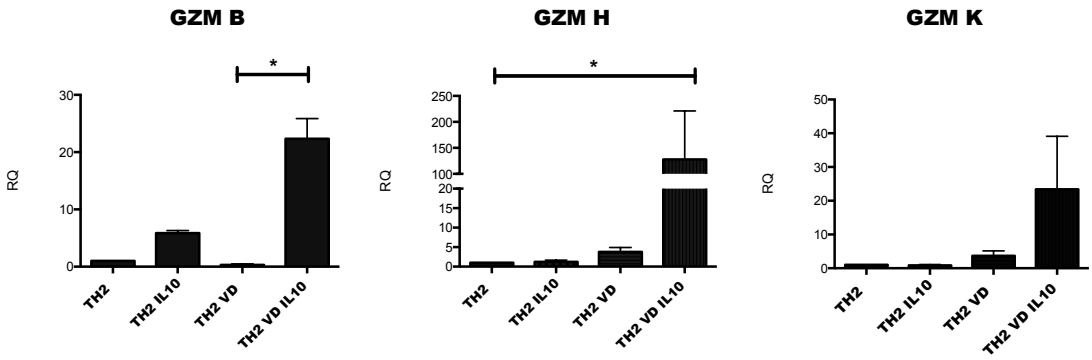
The transcriptome results were further interrogated by analysis of granzyme gene expression in the different cell lines and this demonstrated highest expression in VDIL-10 cells, GZMB and GZMH in particular ($p<0.05$).

Figure 4.7 Analysis of expression of genes involved in Treg immune response in the Th2 lines and the drug deviated cell lines.



Cells were cultured under Th2 deviating conditions, some in the presence of IL-10 (Th2 IL-10) or with addition of 1α,25[OH]$_2$D3 plus dexamethasone (both at 10^{-7}M) from day 14 of culture. Cell pellets were obtained at d28 and mRNA was extracted from the cell pellets. Quantitative rT-PCR was performed using cDNA created from the mRNA . Data represents mean + SEM of 3 experiments performed using blood from 3 individual donors, one way ANOVA

Figure 4.8 Analysis of granzyme expression in the Th2 lines and the drug deviated cell lines.



Cells were cultured under Th2 deviating conditions , some in the presence of IL-10 (Th2 IL-10) or with addition of 1α,25[OH]$_2$D3 plus dexamethasone (both at 10^{-7}M) from day 14 of culture. Cell pellets were obtained at d28 and mRNA was extracted from the cell pellets. Quantitative rT-PCR was performed using cDNA created from the mRNA. Data represents mean + SEM of 3 experiments. performed using blood from 3 individual donors. * $p < 0.05$, one way ANOVA

4.3 Discussion

Calcitriol has significant effects on genes related to both innate and adaptive immunity, and a large number of genes that are involved in innate immunity pathways were expressed in preliminary work from the lab performed on freshly isolated peripheral blood CD4 cells treated with calcitriol and dexamethasone, including TLR9, TLR2 and SERPINA. On review of mRNA expression in Th2 cells deviated with drugs, decreased expression of these genes was noted on qPCR analysis of mRNA expression in the Th2 cell lines alone and also in the Th2 cell lines deviated with drugs (with the exception of TLR2). One possible explanation for this may be the cytokine environment surrounding the cells. The Th2 cell lines were driven by addition of exogenous IL-4 to culture, and IL-4 can suppress some TLR responses, including TLR9[164,165]. SERPINA codes for one of the serpin proteins, which are serum protease inhibitors. Serpin activity has been characterised in the context of alpha 1 antitrypsin deficiency and chronic obstructive pulmonary disease, involving excessive neutrophil elastase activity. The role of serpins in T lymphocytes is not very well defined, and the data from my experiments do not show a sufficiently strong trend to allow conclusions to be made in relation to the regulation or any potential function of serpin expression by T cells.
Similarly, although IGF-1 mRNA expression was increased in the Th2VDIL-10 deviated cultures from 2 donors the data are too preliminary to draw any strong conclusions.

The transcriptome analysis of the Exon ST microarray revealed pathways that may be of significance in relation to the function of the deviated regulatory cells. On review of the transcription factors and cytokine analysis described in the previous chapter, the expression of GATA3 was reduced in the deviated cells in comparison to the effector cells, although not completely abrogated, with a moderate fold change noted. There was reduced mean expression of RORC in all cell lines in comparison with the Th2 cytokine associated transcription factors. The same effect was seen for TBX21 (T bet). Variability in Foxp3 mean intensity levels between conditions was seen in parallel with the mRNA findings described in the previous chapter, i.e. higher in VD than

VDIL-10 or Th2. Reassuringly IL-10 analysis showed high levels and high fold change in the VDIL-10 group compared to Th2 and VD groups.

The transcriptome analysis of the Exon ST microarray revealed pathways that may be of significance in relation to the function of the deviated regulatory cells, with the increased expression, validated through PCR, of a number of genes related to immune function and also genes related to cytotoxic cell killing.

BTLA (B and T lymphocyte activation factor) was evaluated as well as PDCDLG2 the gene for PD ligand 2, one of the ligands that binds PD-1, a marker of Treg function and immunosuppression. BTLA is an inhibitory receptor that has homology with CTLA-4 and PD-1 [166] and binds HVEM (the TNF receptor herpesvirus entry mediator). PD-L2 is one of the ligands for the PD-1 molecule and belongs to the B7:CD28 superfamily of ligands. PD-L2 predominantly acts via PD-1 but may have anti-inflammatory actions that are PD-1 independent, preventing eosinophilia[167] and promoting respiratory tolerance[168]. Fold change of gene expression between Th2VDIL-10 and Th2 cell lines was notable, and there was higher expression seen in the VDIL-10 cells on validation of mRNA, although the increase did not achieve statistical significance. Further biological replicates would need to be studied to determine whether this represents a reproducible trend, and whether these molecules are implicated in the suppressive function of the deviated Th2 cells.

The transcriptome analysis of the Exon ST microarray revealed pathways that may be of significance in relation to the function of the deviated regulatory cells, with the increased expression, validated through PCR, of certain granzymes (B, H and K). The highest level of granzyme expression seen related to granzyme H, although previous authors have noted a link between IL-10 and granzyme B expression. Human IL-10 Tregs that suppress Th1 responses have been noted to express granzyme B[160,169]. Granzyme B knock-out mice have impaired natural Treg function[170]. Tregs regulate

immune responses to viral infection in the lung via a granzyme B mechanism [161].

Granzyme B acts via cell-to-cell contact with effector T cells [147] . Its action is typically facilitated through the action of perforin to enable transfer to the cell targeted for lysis. Tr1s may also kill myeloid APCs through a granzyme B mediated process leading to bystander suppression [147]. Granzyme B cell killing is classically caspase 3 dependent although it may also occur independently of caspase-3 [147]. The action of Granzyme H is also caspase independent [171]. Thus Th2 cells deviated with calcitriol, dexamethasone and exogenous IL-10 appear to possess more than one inhibitory mechanism that can dampen down unwanted immune responses associated with allergic disease. A priority for future work would be to validate the protein expression of these various molecules by flow cytometry and/or antibody capture assays, and ideally to investigate whether the deviated Th2 cells used these molecules to exert their inhibitory activity.

The last experimental aim of the chapter was to validate the mRNA analysis by exploring the action of postulated genes/proteins by blocking studies *in vivo*. From the chapter outlined above, a robust target would appear to be granzyme activity, given that there was high fold change between Th2VDIL-10 and Th2, as well as Th2VDIL-10 and Th2VD cells. Time available precluded further work on mechanistic function of the granzymes, but this would be a central focus for this work to be progressed further.

Experimental approaches would initially include visualisation of granzyme protein expression by flow cytometry[161] and/or microscopy. However, the major priority of future studies would centre around assessing whether granzymes play a role in the inhibitory function of deviated Th2 cells. Approaches would include:

i. Assessment of cell death using traditional methods such as annexin staining to denote apoptosis and propriidium iodide to denote necrosis, in combination with flow cytometry.

ii. Inhibition of specific granzyme activity using blockers such as compound 20[172], which blocks granzyme B function. Bioluminescence assays could also be used that would involve the use of luciferase biosensors linked to specific granzyme cleavage sites[173], that would be activated by granzyme in target cells and thereby monitor specific granzyme activity in co-culture.

iii. Measuring downstream activity to determine if regulatory activity is caspase-dependent by assessing the level of active caspase 3 by flow cytometry[174], or granzyme interactions that are caspase independent, such as the rate of bid (proteolytic enzyme) cleavage, or the effect on cytochrome c. In is noteworthy that granzyme H activity may be caspase, bid and cytochrome c independent[175,176].

Chapter 5

Steroid insensitivity and Th17 cytokines - assessment of IL-17 responses in CD4 cell cultures and the effect of calcitriol and dexamethasone

5.1 Introduction

The previous chapters have described the plasticity and stability of Th2 cell phenotypes. Typically asthmatic and allergic responses are characterised by early and late phase responses dominated by Th2 cytokines[71]. Whilst the majority of patients respond favourably to steroids for control of their clinical symptoms in association with downregulation of the Th2 response, there exists a significant cohort of patients with significant morbidity related to their asthma who exhibit insensitivity, or resistance to steroids[177]. The previous chapters studied the capacity of steroids, together with the steroid response modifier, vitamin D[131], to influence the Th2 immune profile towards a more immunoregulatory profile. Alternative T cell pathways may influence steroid insensitivity, in particular Th17 mediated pathways[178].

The T helper cell family has expanded with the discovery of Th17 cells. These cells are differentiated in the presence of IL-6, IL-21 and IL-23 in humans, and they are thought to play a role in defence against extracellular pathogens such as fungal organisms and extracellular bacteria at mucosal sites such as the airways[179]. The release of Th17 cytokines promotes expression of proteins that are responsible for innate defences such as defensins[9]. Th17 cells co-express IL-22, although work has demonstrated another category of CD4+ Th22 cells with a distinct cytokine and profile, that express IL-22 independently of Th17 cells and may be responsible for modulating pathology in dermatological disease[180]. Th17 cells may play a role in combating infections with extracellular pathogens, but uncontrolled expression of IL-17 may contribute to autoimmune diseases such as rheumatoid arthritis and ulcerative colitis.

IL-17 expressing cells have been noted in mild as well as severe asthma[181,182], but studies suggest that IL-17 positive cells may contribute towards a more severe asthma phenotype. IL-17+ve cells have been noted in the bronchial tissue of asthmatics with increasing frequency seen in severe disease[183,184], in addition to increasing levels of IL-17 mRNA in sputum[185].

One group demonstrated that increased levels of IL-17 in serum correlated with asthma severity[186].

Studies have been performed to try and identify inflammatory phenotypes within cohorts of patients with severe asthma. Steroid insensitivity in some cases may be associated with inflammatory profiles that lean towards neutrophilic rather than eosinophilic inflammation[177]. IL-17 production can be associated with a steroid-resistant phenotype[187], with evidence in humans that it may promote neutrophilia in the airways[188 185].

Earlier bronchial studies had suggested that eosinophils could be a possible source of IL-17 in the bronchi, at least in mild asthmatics[182], with increased IL-17 levels seen in the presence of significant sputum eosinophilia in patients with less severe disease, but other authors have noted that the T cells are the source of IL-17, particularly in more severe asthmatics[185]. IL-17 production may also contribute to remodelling as it promotes the proliferation of human fibrocytes[189]. Mechanistic studies suggest that Th17 cell phenotypes can skew towards a more pathogenic phenotype associated with IFN gamma production[190], with certain pathogens promoting a Th17 and neutrophil response within asthmatic airways[191]. This response may be amenable to macrolide treatment, at least in animal models[192].

Calcitriol has varying effects on Th2 cytokines – it may augment Th2 cytokine production in CD4 culture[120,193] and previous work has demonstrated that it augments IL-10 production in CD4+ T cells[131].

Hypothesis

In the previous experimental chapters my work identified a potential role of vitamin D plus dexamethasone (and IL-10) in beneficially modifying Th2 responses in the context of allergy/asthma. However additional/more recent work highlights a role for Th17 cells in asthma pathology, of interest and importance since Th17 responses are proposed to be of important in steroid refractory disease. The hypothesis to be investigated here, therefore, is that Th17 responses will show a distinct profile of modulation by dexamethasone plus calcitriol in comparison to the previously observed and reported effects on Th2 responses.

Experimental aims

The aims of the experiments described in this chapter were as follows:

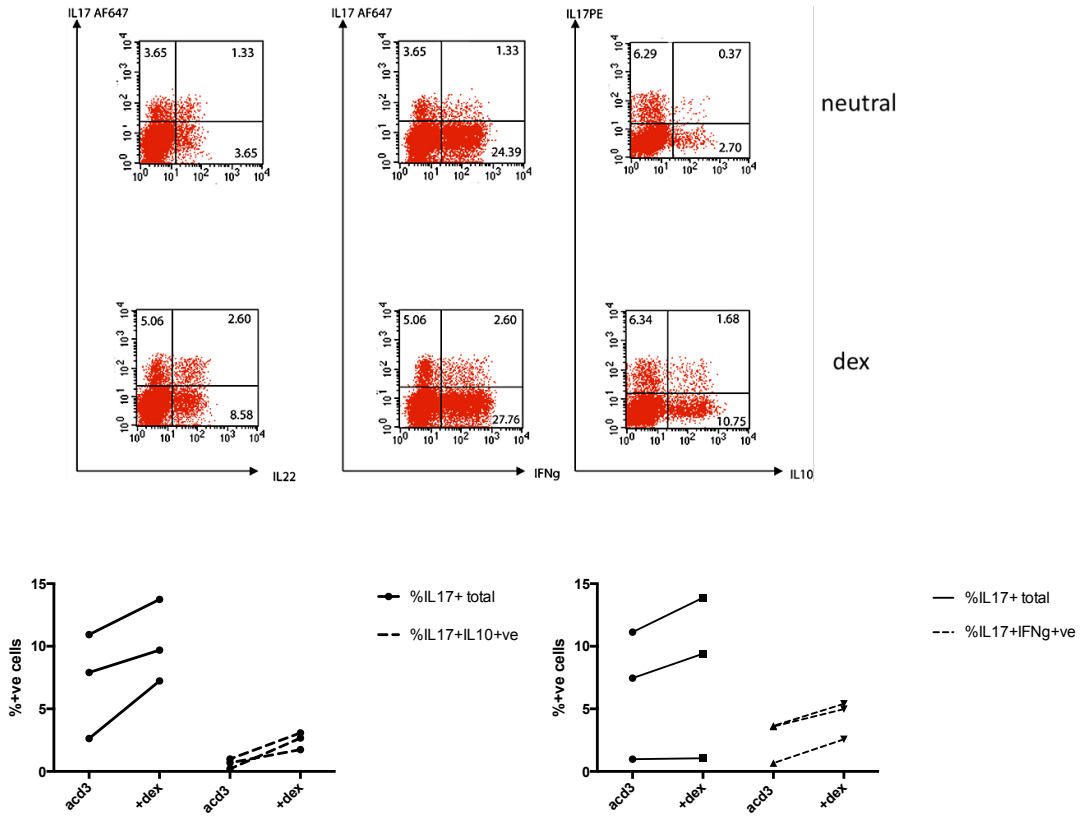
- To assess the effects of dexamethasone on Th17 cytokines.
- To assess the impact of different T cell stimulation conditions on production of Th17 cytokines in relation to dexamethasone, with anti-CD3 and low dose IL-2 initially, and then against the more powerful stimulus of anti-CD3 plus anti-CD28; followed by investigation of Th17 cytokine production in the presence or absence of IL-4 within culture.
- To assess the effect of vitamin D, in the presence or absence of dexamethasone, on IL-17A production within this T cell model.

5.2 Results

5.2.1 Dexamethasone maintains CD4+ IL-17+ and IL-22 + populations, and may promote inflammatory IL-17A responses.

CD4+ T cells were isolated from peripheral blood mononuclear cells using magnetic beads and cultured for 7 days. TCR stimulation was achieved by anti-CD3 stimulation alone without the use of anti-CD28 or antigen presenting cells. Cells were cultured in the presence of low dose IL-2, with and without 10^{-7}M dexamethasone, from day 0 of culture.

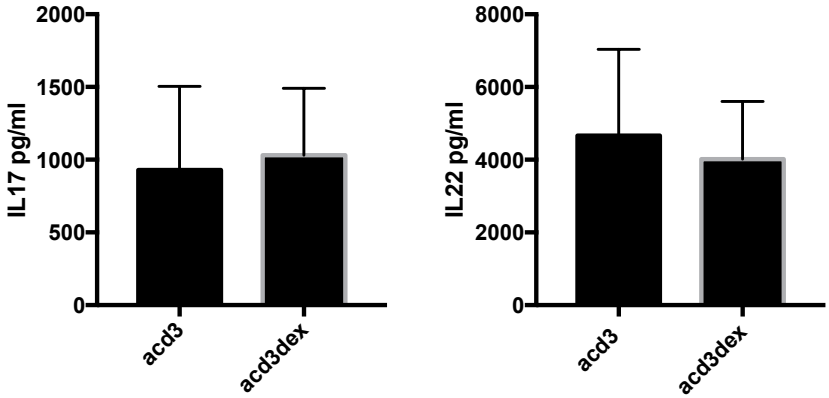
Figure 5.1. Intracellular cytokine staining profile following treatment of CD4+ T cells with dexamethasone.



Top figure. CD4+ T cells were isolated with magnetic beads and cultured for 7 days with αCD3 and IL-2 with dexamethasone 10^{-7}M (dex) or without (neutral). Intracellular cytokine staining was performed following stimulus with PMA and ionomycin at day 7. Representative experiment of 3 shown
Bottom figure. Analysis of staining – review of total versus double positive cells. CD4+ T cells were isolated with beads and cultured for 7 days with αCD3 and IL-2 with or without dexamethasone at 10^{-7}M from day 0. Intracellular cytokine staining analysis was performed at day 7 following stimulus with PMA and ionomycin. Left – total IL-17A+ vs. IL-17A+IL-10+ cell frequency, n=3, 3 individual donors. Right – total IL-17A+ vs IL-17A+IFNγ+, n=3, three individual donors.

Analysis of the cell populations demonstrated that cultures containing dexamethasone produced more IL-17+ and IL-22+ cells than the neutral condition (absence of dexamethasone; Figure 5.1), with almost double the number of single positive IL-17A+ cells seen via intracellular cytokine staining, but also an increase in IL-17A+IL-22+ populations, in addition to IL-22+ single populations. However, in the small number of experiments performed (n=3) this did not achieve statistical significance and is therefore best described as the failure of dexamethasone to inhibit the IL-17A response or a potentially a trend to enhance this response. Comparable levels of IL-17+ and IL-22+ secretion were seen when analysing cytokines via ELISA (Figure 5.2), in cultures irrespective of whether dexamethasone was present in culture. On further review of the cytokine profile, culture of the CD4+ T cells with dexamethasone showed a trend for an increase in the proportion of IL-17A+IFNγ+ cells, and IL-17+IL-10+ cells, but these were proportionately fewer than the single positives (Figure 5.1). The level of single positive IL-10+ve cells increased as expected with dexamethasone (Figure 5.1).

Figure 5.2. ELISA analysis of IL-17A and IL-22 levels after treatment with dexamethasone.

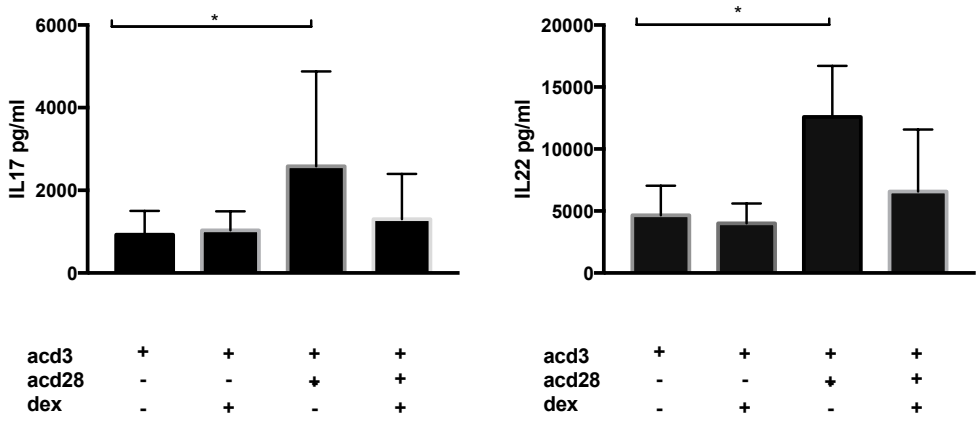


CD4+ T cells were isolated with magnetic beads and cultured for 7 days with anti-CD3 (αCD3) and IL-2 with or without dexamethasone (dex) at 10^{-7}M from day 0. Cell supernatants were obtained and an ELISA was performed to assess cytokine levels at day 7. N=3, three individual donors, data = mean + SEM, p=ns, student t-test.

5.2.2 Effect of stronger T cells stimulation on Th17 responses

The maintenance, and enhancement of IL-10 expression with dexamethasone may reflect the effect of low dose IL-2, and a mild T cell growth stimulus of CD3 alone, in the absence of αCD28 stimulation. although this can also promote IL-17A expression, with the generation of a mixed population of cells co-expressing IL-17A plus IFNγ, IL-17A plus IL-22 and IL-17A plus IL-10 (Figure 5.1). The next set of experiments aimed to assess the effect of a more powerful T cell stimulus with the addition of costimulatory anti-CD28 in culture together with anti-CD3.

Figure 5.4. Assessment of TCR stimulation with aCD28 vs effects of dexamethasone



CD4+ T cells were isolated with magnetic beads and cultured for 7 days with anti-CD3 and IL-2 with or without anti cd28, or dexamethasone 10^{-7}M (dex) from day 0. Cell culture supernatants were obtained and an ELISA was performed to assess cytokine levels at day 7. Data = mean of 3 experiments (3 individual donors) + SEM, *p<0.05, one way ANOVA.

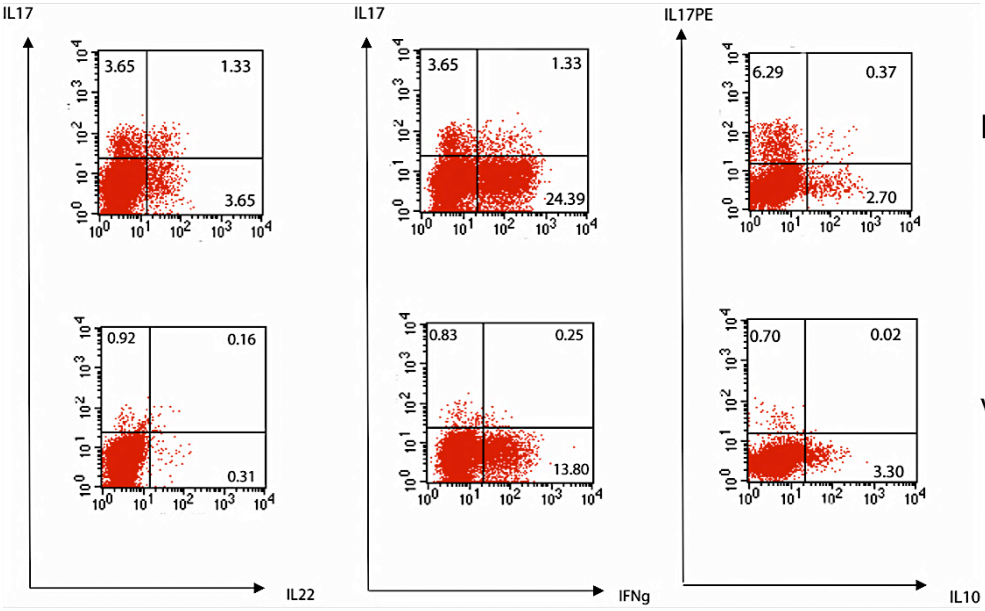
After 7 days of culture it was evident that total IL-17 and IL-22 production was increased with the stronger stimulus of anti CD3/anti CD28 as compared to anti CD3/low dose IL-2 in 'neutral' cultures with receptor stimulation alone and without any dexamethasone (Figure 5.4). Interestingly the level of IL-17A production was not maintained with dexamethasone when cells were cultured with the drug in the presence of anti-CD28 with anti-CD3 stimulus, as

previously seen with anti-CD3 plus low dose IL-2 stimulus. The same effect was seen for IL-22 synthesis after 7 days of culture (Figure 5.4).

5.2.3. The frequency of CD4+ IL-17+ and IL-22 + populations is reduced by the presence of calcitriol in cell culture.

Vitamin D modulates a number of cytokines with variable effects. It may enhance Th2 cytokines before diminishing their production by T cells in long-term cultures as well as enhancing production of more regulatory cytokines such as IL-10[39,120,193]. The next set of experiments was performed to assess the effect of vitamin D on IL-17A when used alone, and to assess the effect of this drug in CD4+ cultures *in vitro* in contrast to, and in combination with, dexamethasone.

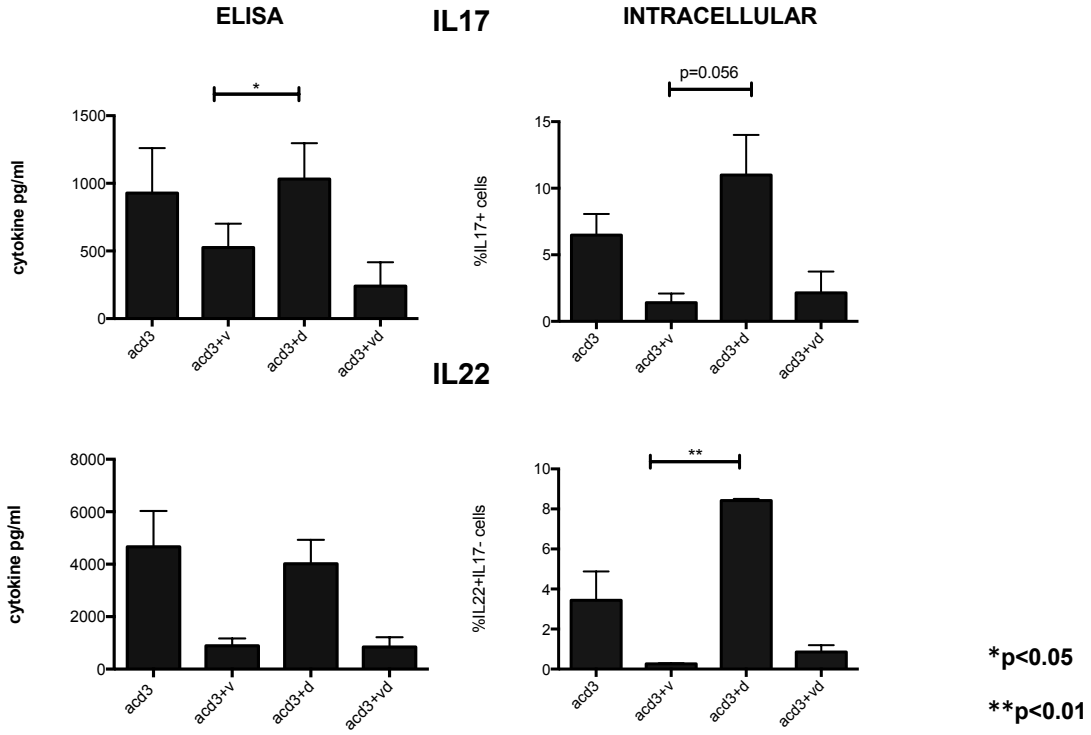
Figure 5.5. Assessment of IL-17A levels in CD4+ T cell cultures following culture with calcitriol.



CD4+ T cells were isolated with magnetic beads and cultured for 7 days with anti-CD3 and IL-2 with calcitriol at 10^{-7}M (vit) or without (Neutral) from day 0. Intracellular cytokine staining was performed following stimulus with PMA and ionomycin at day 7. Representative plot from 3 experiments.

CD4+ cells were cultured for 7 days in the presence of anti-CD3, and IL-2 with and without calcitriol. There was a marked reduction in most of the cytokines produced at day 7 in comparison to the neutral condition, with marked attenuation of the IL-17A and IL-22 responses. The IFNγ response was reduced but this was less profound. There was preservation of the IL-10 response (Figure 5.5).

Figure 5.6. Assessment of effect of calcitriol and dexamethasone on IL-17 production



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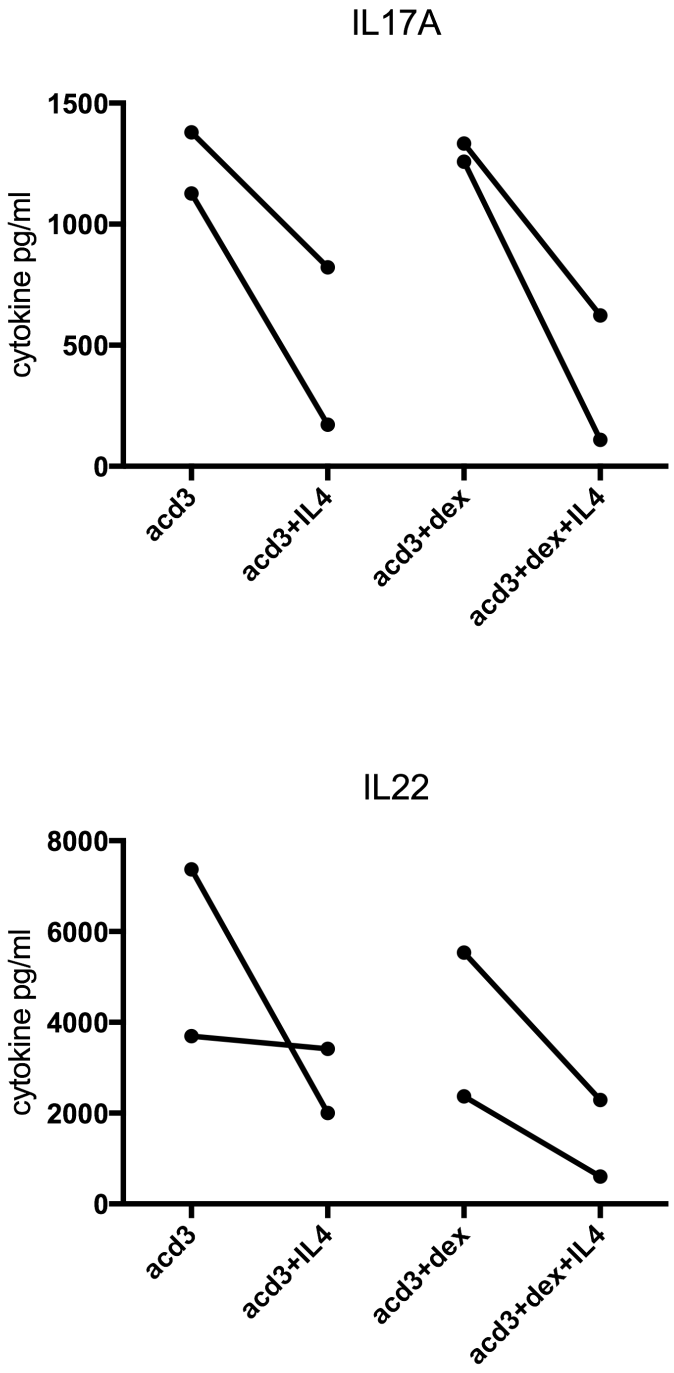
CD4+ T cells were isolated with magnetic beads and cultured for 7 days with anti-CD3 and IL-2 with or without calcitriol (v) and/or dexamethasone (d; vd) at 10^{-7}M from day 0. The supernatant from cells isolated at day 7 was analysed using ELISA. Intracellular staining was performed following stimulus of the cells with PMA and ionomycin at day 7. Data represents mean of 3 experiments (3 individual donors) + SEM, *P<0.05, p<0.01 one way ANOVA

The ELISA data (Figure 5.6) showed a marked decrease in IL-17A and IL-22 cytokine levels with the addition of calcitriol *in vitro*. Dexamethasone alone failed to significantly alter IL-17A or IL_22 secretion under these culture conditions. On analysis of the combined effects of the drugs, it was noted that the diminution in IL-17A and IL-22 responses was maintained when calcitriol was used in combination with dexamethasone, thus the inhibitory effects of calcitriol on Th17 cytokine responses appear glucocorticoid independent.

5.2.4. The Th2 cytokine, IL-4 diminishes CD4+ IL-17+ cell responses to dexamethasone in culture

The characteristic cytokine that defines and promulgates the Th2 phenotype is IL-4, with reciprocal downregulation of Th1 responses. The CD4+ culture system outlined above was interrogated to identify if IL-4 exhibited any influence on the Th17 cytokines when combined with dexamethasone *in vitro* (Figure 5.7).

Figure 5.7. Assessment of effect of IL-4 on IL-17 production



CD4+ T cells were isolated with magnetic beads and cultured for 7 days with anti-CD3, IL-4 and IL-2 with or without dexamethasone 10^{-7}M from day 0. The cell supernatants were isolated at day 7 and analysed using ELISA. Results for two separate donors shown

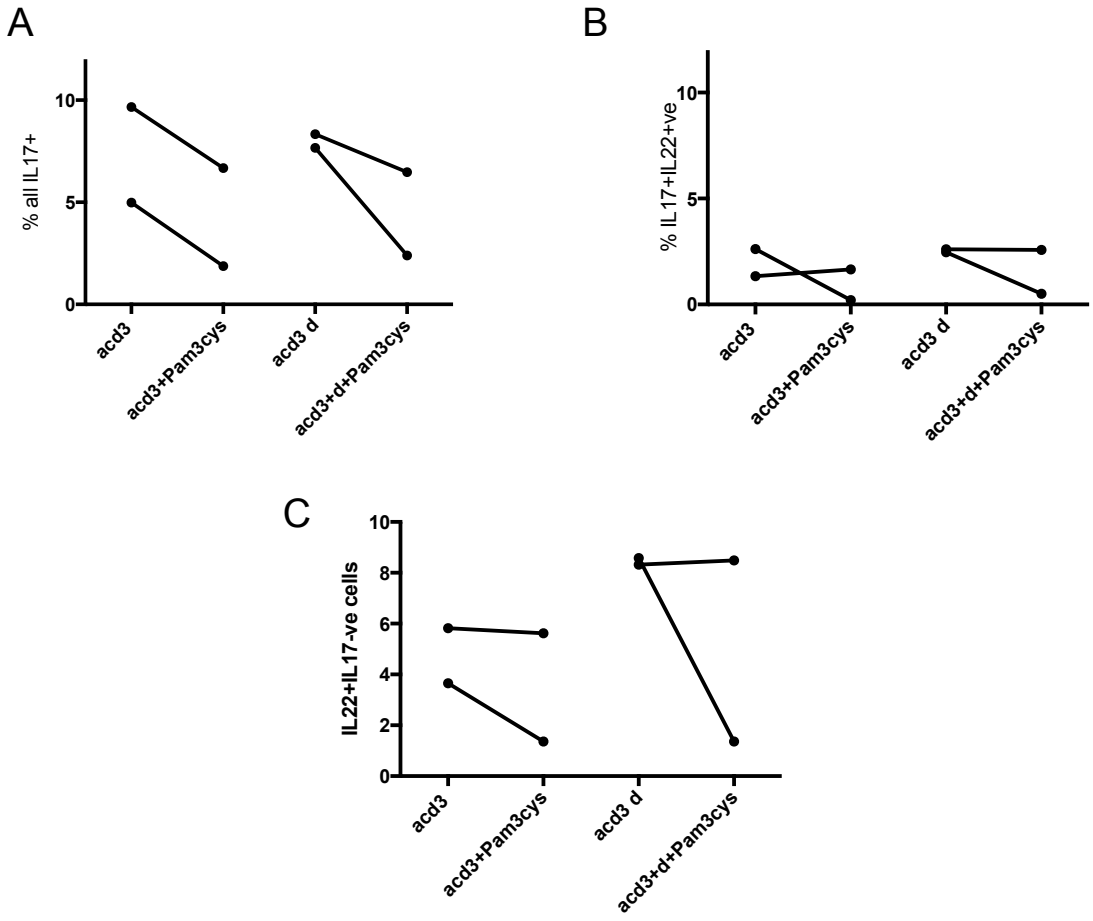
Experiments demonstrated that the addition of IL-4 to the cell culture reduced the levels of IL-17A and IL-22 overall in the presence of the neutral condition

(stimulus with anti-CD3 alone in the presence of IL-2). The previous observation seen with dexamethasone in culture, namely maintenance of IL-17A and IL-22 levels at day 7 of culture of CD4s in the presence of dexamethasone was not sustained and levels also decreased (Figure 5.7) in the presence of IL-4, although the experiments were performed for only two donors and therefore it would be difficult to draw very strong conclusions from the data.

5.2.5. Pam3 Cys, a TLR2 agonist, does not augment IL-17A responses in the presence of dexamethasone

A recent paper [194] indicated that stimulation of the TLR2 receptor on CD4+ T cells, in a mouse model may augment IL-17A responses. This was proposed to facilitate the augmentation of IL-17A and IL-22 responses that promote innate immunity. Work previously performed in our lab demonstrated that the addition of calcitriol and dexamethasone to CD4+ T cell culture augments the expression of the TLR2 receptor and further work demonstrated that dexamethasone alone increases TLR2 expression (unpublished data). The next set of experiments was performed to assess if the addition of a TLR2 agonist, Pam3Cys, to culture of human CD4+ T cells with dexamethasone would augment the IL-17A response.

Figure 5.8. Effect of TLR2 antagonist, Pam3cys, on IL-17 and IL-22 expression in culture with dexamethasone



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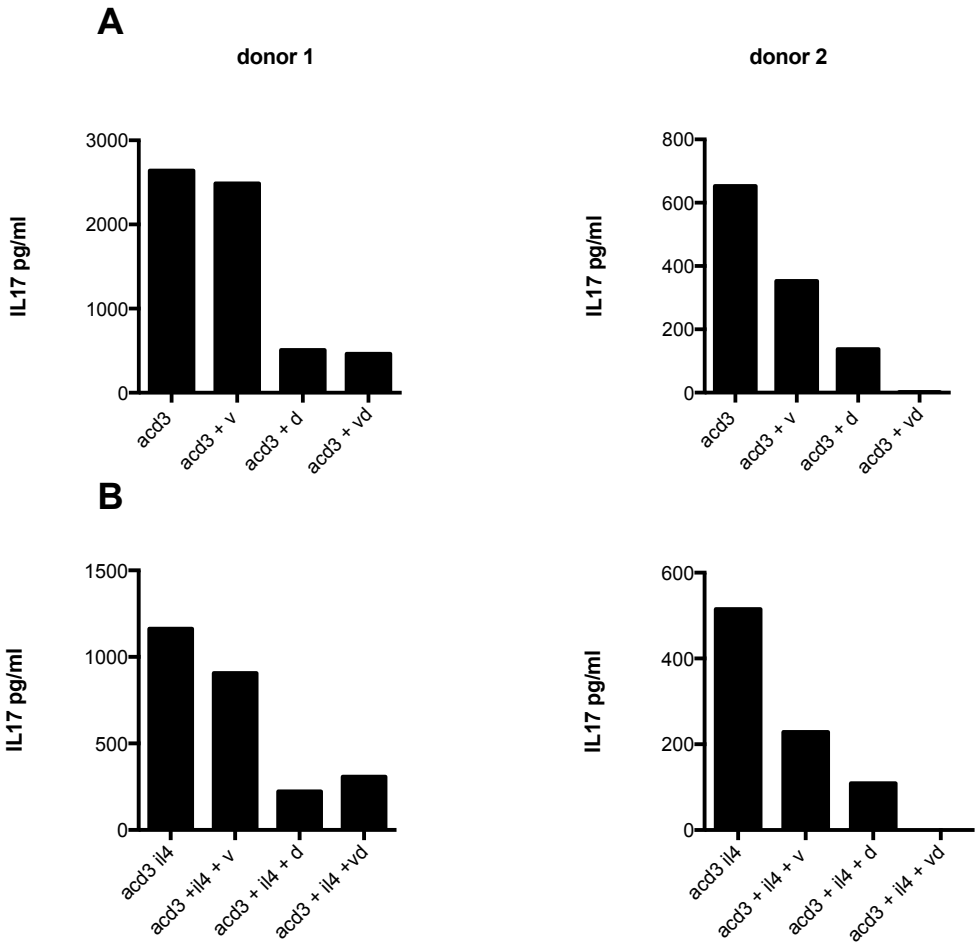
CD4+ T cells were isolated with magnetic beads and cultured for 7 days with anti-CD3, and IL-2 with or without dexamethasone 10^{-7} M (d) and/or Pam3Cys from day 0. Intracellular cytokine staining analysis was performed at day 7 following stimulation with PMA and ionomycin. A– IL-17A; B IL-17+IL-22+ cells. C – IL22+IL17- cells. Data from two separate donors shown.

Analysis of IL-17A levels by intracellular staining showed that IL-17A levels were reduced in the presence of Pam3Cys and the TLR2 agonist failed to maintain or enhance IL-17A responses in culture with dexamethasone. A similar pattern was observed when analysing the IL-22+ single positive population (Figure 5.8).

5.2.6. Assessment of cytokine production at earlier timepoints suggest that higher levels of IL-17A are seen with vitamin D rather than dexamethasone at day 3 compared to day 7

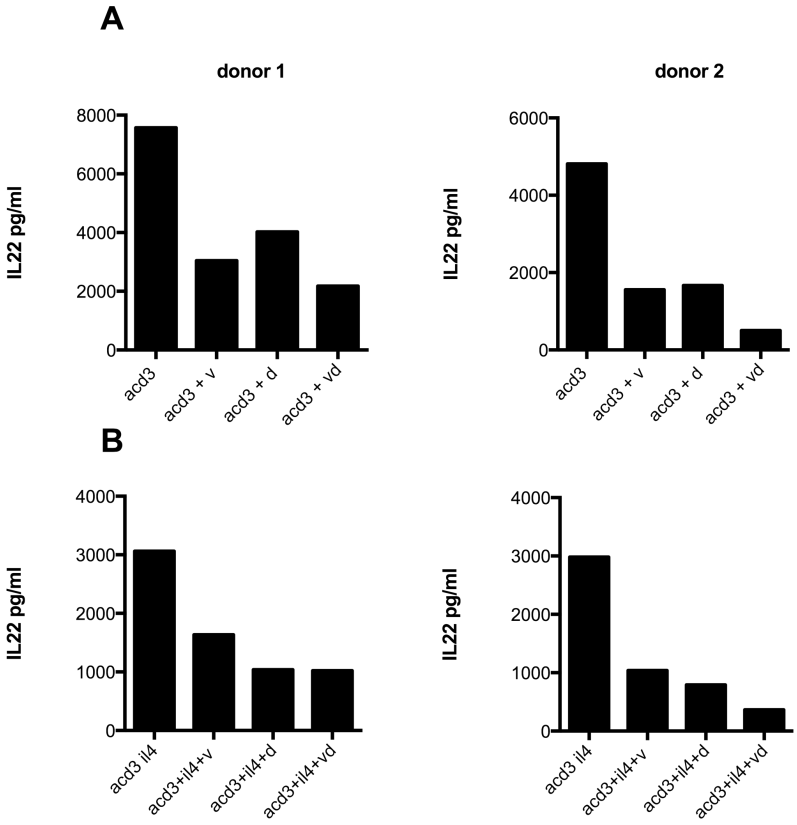
The kinetics of IL-17 responses were further investigated by assessing the profile of ELISA data for cytokines produced at day 3 of culture with anti-CD3 (with and without drugs, and also with and without IL-4). Interestingly, in contrast to the high levels of IL-17 seen in culture with dexamethasone at the day 7 timepoint, (Figures 5.1 and 5.2), higher levels of IL-17 and IL-22 were seen in the presence of calcitriol at day 3 than with dexamethasone(Figure 5.9). All levels were reduced in the presence of IL-4 (Figure 5.9B and 5.10B) as seen previously in the culture at day 7.

Figure 5.9. Assessment of effect of dexamethasone and calcitriol on IL-17 production at the earlier time points of day 3



CD4+ T cells were isolated with magnetic beads and cultured for 3 days with anti-CD3 (acd3), IL-4 (il4) and IL-2 with or without dexamethasone 10^{-7}M (d) and/or calcitriol (v; vd) as indicated. The supernatant from cells isolated at day 3 was isolated and analysed using ELISA. A – IL-17 without and B IL-17 with IL-4 in culture. Data from 2 separate donors is shown.

Figure 5.10. Assessment of effect of dexamethasone and calcitriol on IL-22 at the earlier time point of day 3



CD4+ T cells were isolated with magnetic beads and cultured for 3 days with anti-CD3 (acd3), IL-4 (il4) and IL-2 with or without dexamethasone 10^{-7}M (d) and/or calcitriol (v; vd) as indicated. The supernatant from cells isolated at day 3 was isolated and analysed using ELISA . IL-22 without (A) and (B) with IL-4 addition to culture. Data from two individual donors is shown.

5.3. Discussion

This chapter assesses the capacity of peripheral blood CD4+ T cells to synthesize Th17-associated cytokines, and the maintenance of IL-17A production in the presence of glucocorticoids in culture.

The experiments were performed on CD4+ T cells freshly isolated from PBMCs and experiments performed *in vitro* showed that after 7 days of culture with a moderate strength T cell stimulus, in the presence of IL-2, that dexamethasone, a glucocorticoid maintained the IL-17+ population. It is likely, given the starting population that the majority of the IL-17+ cells were CD45RO+ rather than naïve CD4+ Tcells[195], an observation that has subsequently been confirmed by others in the lab (E Mann, unpublished data). Dexamethasone also maintained the IL-22+ve population in addition to the total IL-17+ve population and there was also a trend towards an increase in the IL-17+IL-22+ population.

The presence of other cytokine double positive populations within the IL-17+ve population was also investigated, as different double positive populations have been postulated to show either pathogenic or more regulatory effects. IL-17+IFNγ+ cells have been identified in murine studies of experimental autoimmune encephalomyelitis[5] and have also been identified in humans[196] where they may cause pathology such as inflammatory bowel disease[197]. Conversely, double positive cells may have a more regulatory phenotype with an IL-17+IL-10+profile.

On review of the cytokine profile of the double positive cells seen on culture of human CD4+ T cells with anti-CD3 with or without dexamethasone, the use of dexamethasone *in vitro* led to expression of both IL-17+IFNγ+ and IL-17+IL-10+ve in equal measure, which indicates that this drug may promote inflammatory responses in addition to the anti-inflammatory actions that are seen in treatment of autoimmune disease. The persistence of a putatively pathogenic population in culture with dexamethasone may account for subsequent resistance to glucocorticoid treatment in chronic disease.

However, the proposed pathogenic or regulatory function of these populations was not assessed, and future work to address this would be important to understand the relevance of these observations. This would include the use of more advanced multicolour flow cytometry to better define the IL17+ve populations, isolation of the different cell populations by cell sorting and coculture experiments with autologous cells to assess proliferation and cytotoxicity.

The effect of a stronger stimulus in culture, namely by combining anti-CD3 with anti-CD28 costimulation, was compared to stimulation with anti-CD3 and IL-2. Results from experiments performed with anti-CD28 *in vitro* demonstrated a higher level of IL-17 production overall in the neutral condition compared to anti-CD3 alone. However, the elevation in IL-17 levels was not maintained in the presence of dexamethasone when anti CD28 was also present in culture, an observation that has again subsequently been validated by others in the Hawrylowicz lab. This may reflect a lack of cytokines that promote the persistence of the Th17 phenotype in the presence of stronger stimulation, such as IL-1β and IL-23[9]. It is notable that both the anti-CD3 and anti-CD28 experiments were performed in the presence of low dose IL-2, which appears to be required for persistence of IL-17 secretion, although this effect is not seen at the onset of culture, and influences phenotype from day 3 and onward[195]. One further step that would help to progress investigation of the influence of dexamethasone at this stage would have been separate experiments with and without IL-2 at early and later time points to assess any differential effect.

The effect of calcitriol was reviewed at two different time points, both at day 3 and day 7. By day 7 of culture with anti-CD3 and IL-2, IL-17 and IL-22 production in the CD4+ T cells was abrogated, with preservation of IL-10 secretion. Previous work has shown that the addition of calcitriol to dexamethasone promotes an IL-10 phenotype and the use of calcitriol and dexamethasone in tandem did not lead to an increase in IL-17 secretion, therefore promoting a more regulatory cytokine pattern. The reduction in IL-17 and IL-22 production by calcitriol is not seen at the earlier timepoint of day 3.

The reason for this was not ascertained in the present study. However, since both calcitriol and dexamethasone are known to reduce cell proliferation, assessment of cell proliferation using a dye such as CellTraceViolet would allow analysis of whether the increase of Th17 cytokine expressing T cells seen in the presence of dexamethasone reflected a preferential expansion of this population as seems likely, and might reflect the relatively late time point at which this effect was observed.

The effect of a TLR2 ligand was studied as it has been reported to augment IL-17 responses when CD4+ T cells are stimulated directly via this receptor [194].In the current experiments with Pam3Cys, a TLR agonist, did not lead to an increase in IL-17A production in the presence or absence of dexamethasone. Other studies looking at the effect of Pam3Cys in human systems have noted that stimulation of Pam3cys augments IL-17 production via the action of dendritic cells that produce cytokines such as IL-6 and IL-1β that stimulate CD4+ cells towards production of Th17 cytokines [195,198]. Dendritic cells may also produce these cytokines in response to TLR4 stimuli (LPS) and imidazoquinolone, a TLR8 agonist[195]. A further extension of this work would be to assess the response to drugs in a system that involved APCs and the different TLR stimuli, which would have relevance for the lung in exposure to different antimicrobial challenges.

This work was commenced as a logical extension to the Th2 study as Th2 and Th17 cells are associated with different phenotypes of asthmatic disease, and the potental for calcitriol and dexamethasone to have differential effects was not anticipated. More detailed cytokine secretion studies would have been of benefit to address the time course of the changes effected by the addition of drugs. Although the current body of work is in many ways incomplete this body of work then merged and was superceded by other ongoing studies in the lab after identifying significant differences in IL-17A in steroid sensitive vs steroid-resistant patients [199] and that an *in vivo* correlate between inhaled steroid dose and IL-17A levels in culture were positively correlated.

Chapter 6

Final Discussion

There is a large body of evidence that outlines the significant burden of asthma and allergic disease in the UK and worldwide. These conditions cause significant impairment in quality of life on a personal level, but also to the general population through time off school or work with subsequent loss of productivity in part due to their very high prevalence. In the UK, it is estimated that around 5.4 million people are currently receiving treatment for asthma i.e. one in every 12 adults and one in every 11 children.

A number of therapies are available to treat allergic disease, the majority of which control disease symptoms but do not cure or prevent long term disease progression. However, the long term immunological goal has always been to identify ways to cure. A number of biological therapies have been investigated including anti-IgE and a number of strategies developed to target cytokines implicated in disease pathogenesis. However, these have largely been disappointing and none suggest that individually that they will provide a long term practical strategy to afford long term relief of disease symptoms. What they have highlighted is the heterogeneity of human allergic and asthmatic disease, and that the quest for improving therapeutic strategies needs to take this diversity into account. A clear example of this are studies of the monoclonal antibody Lebrikizumab (anti-IL-13) which only improved lung function in patients with a periostin-high asthma phenotype, an observation that encouragingly originated in studies of molecular phenotype[84].

Allergy, uniquely, has had an antigen-specific treatment in use, pioneered over 100 years ago i.e. allergen immunotherapy; however this is not commonly advocated for allergic asthma in the UK, although widely used overseas, as it can poses significant risks and is not unilaterally effective. It is generally assumed, particularly in studies of allergic rhinitis, to require a prolonged period of treatment to gain maximal efficiency, which can make it cumbersome to administer.

A number of strategies are being studied to improve both the safety and efficacy of allergen IT. These include modified allergens (e.g. peptides, chemically modified allergens), different routes of delivery, as a combination

therapy for example with anti IgE, and adjuvants that may enhance the capacity to promote regulatory pathways such as IL-10 since good evidence exists that where IT is effective this is associated with increased IL-10 synthesis within the monocyte, B and T cell compartments.

There has been a lot of evolving work in the past decade, reflecting the complexity of T cell plasticity, with additional T cell subsets described that are proposed to play a role in allergic and asthmatic disease beyond the original Th2 paradigm[187] as well as evidence for the potential plasticity between T cell subsets, including both regulatory T cells and effector subsets. Allergen immunotherapy, generally used to target Th2-mediated allergic disease, may lead to deviation of a Th2 response towards a more regulatory phenotype[140]. The broader remit of the work presented here aimed to investigate mechanisms that might improve the safety and efficacy of allergen IT, directed particularly at Th2 mediated allergic disease. It sought to build on earlier findings within the scientific field, in part highlighted above and discussed within this thesis, and from our lab that steroids with vitamin D promote IL-10 synthesis. The work was done to establish if deviation of existing Th2 responses associated with disease and effective IT could be enhanced by combining with dexamethasone and vitamin D. The work was subsequently extended to identify the effects of vitamin D and dexamethasone on Th17 responses, associated with a distinct phenotype of severe asthma.

The major findings of this work demonstrated that Th2 cells that were deviated with calcitriol and dexamethasone have a similar profile to Tr1 cells. There is a requirement for exogenous IL-10 but this does not appear to be the mechanism for suppression as this action is not blocked by anti IL-10R (and is not affected by the action of anti TGFb or anti CTLA4). One caveat to this conclusion however, is that the blocking reagents used in this study were only tested singly and not in combination. The deviated cells do not exhibit the phenotype of natural T reg as they exhibit low Foxp3 expression in combination with high IL-10 expression. A pragmatic analysis of the ExonST microarray demonstrated upregulation of genes related to granzyme activity, which may be a putative mechanism for the action of the suppressor cells and

account for ‘regulatory cell’ activity. It has been demonstrated that regulatory cells act through the action of granzyme in other Treg systems, such as those mediated by the action of complement[43], and may also have a role in mediating responses to viral infection, associated with an IL10 response mediated by AhR[161].

The manipulation of Th2 cells to deviate towards a regulatory profile may mean that the use of these drugs is an alternative to standard glucocorticoid therapy and also may be of benefit in immunotherapy to augment accelerate the conversion of Th2 responses towards an immunoregulatory profile. One major caveat is that we have still yet to understand the full capacity and plasticity of T cell phenotypes and we must ensure that the regulatory cells maintain their beneficial phenotype without conversion or rather reversion to the previous harmful Th2 effector phenotypes, which may be harmful.

These experimental studies utilised the drug combination of the glucocorticoid dexamethasone, together with the active form of vitamin D3, 1α,25-dihydroxyvitamin D3 or calcitriol, to effect deviation of a disease-promoting Th2 profile towards a more regulatory IL-10+ phenotype. A more acceptable and practical therapeutic approach to adopt in patients might be to restore vitamin D sufficiency through supplementation of those individuals prior to and during immunotherapy, given the high prevalence of vitamin D deficiency and insufficiency. Supplementation with 25-hydroxyvitamin D3, as opposed to 1α,25-dihydroxyvitamin D3, is likely to be more acceptable, having a longer half life and with fewer potential safety concerns. Furthermore, the capacity of vitamin D to promote and maintain multiple regulatory pathways, including IL-10, is arguably a good conceptual basis for such a strategy. Counter arguments to such an approach include experimental evidence that vitamin D can enhance Th2 responses, although as discussed within this thesis, paradoxically an inverse correlation between vitamin D status and parameters of allergic disease exists[122,123], and this may therefore not reflect such a serious concern. The concept of combining vitamin D treatment with allergen immunotherapy is being increasingly explored in animal models[200-202] which in

general provide positive data to support this strategy together with a few early studies in humans, which provide some supporting data[203,204].

Calcitriol was also found to have additional effects on Th17 cytokine production even in the presence of dexamethasone, with a broader implication for the use of this drug in treatment of (glucocorticoid resistant) allergic disease. The lab subsequently went on to show a comparable modulation of Th17 responses by calcitriol *in vivo*[205]. The messages from that study are a validation of a number of years work from the lab showing that steroids upregulate IL-10, that this response is impaired in steroid resistance and can be restored with calcitriol. However the observation made here and extended through other studies in the lab, that steroids fail to inhibit and can increase IL-17A, an effect that is more marked in steroid refractory asthma, is noteworthy. We have hypothesised that the capacity of steroids to maintain an IL-17 response will play an important role in maintaining protection to mucosal pathogens in mild/moderate disease, although clearly as indicated by both our own and independent work an over exuberant IL-17 response is likely to be detrimental in severe disease. What alters the balance between a putatively protective IL-17 response versus one assocated with disease pathology is unknown. Furthermore whether protective and detrimental IL-17/Th17 responses differ qualitatively, as alluded to in the present studies and other work published form our lab[199] remains to be fully investigated and determined. Importantly evidence from work performed in the lab following on from this thesis shows that vitamin D/calcitriol in culture, and following ingestion of calcitriol by steroid- resistant patients inhibits the Th17 response even in steroid-resistant patients[199].

Further work to try and augment the experiments outlined above should aim to look to generate more allergen specific modes of action and attempt to delineate and refine the mechanisms of action of the deviation towards regulatory responses.

Initially the aim would be to assess the capacity to develop a more allergen-specific model of the Th2 cell lines studied here. Multicolour flow cytometry

has progressed tremendously and this would enable re-evaluation of strategies to establish markers to identify the cell phenotype e.g. CD127, LAG3, CD39 etc. This could be used alongside techniques such as HLA class II peptide tetramers[206] to stain allergen specific T cells, as well as intracellular cytokine staining to evaluate allergen specificity both experimentally in culture and animal models, but also ideally in patients[207].

Cells deviated in the presence of calcitriol and dexamethasone could be investigated to assess stability of the deviated Th2 cells after withdrawal of promoting cytokines over a more prolonged period than that required for the CFSE suppression assay. This capacity for plasticity should be tested in relation to the presence of other stimuli including allergens and Th17 stimuli to assess their capacity to maintain their phenotype and prevent transformation to Th17 phenotypes[208]. Translation of these important issues directly into patient based studies is highly desirable and is now becoming realistic based on ongoing technical advances such as those noted above.

In order to explore mechanism of action of the new deviated cells it would be imperative to examine the capacity if the cells to utilise granzyme and perforin pathways and identify if this is the true mechanism that underlies regulation[171,209].

The Exon ST array could be examined further to identify other genes of interest that would assist in definition of the Treg phenotype. Ideally in order to facilitate analysis, a greater number of arrays would be required for each cell line so that biocomputational review can take place. Whilst a number of limitations exist in the present study, gene transcriptional arrays studies have proved highly successful in identifying new gene targets and biomarkers in allergic disease[72], and the current work has opened up new potential avenues for further investigation.

In conclusion, the work outlined above demonstrates the capacity for immunomodulation using vitamin D3 to combat immune mediated disease and provides hope that a strategy involving treatment with vitamin D3 carries

the potential for improved treatment alone or in concert with other forms of immunosuppression or immunotherapy.

References

1. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986; **136**(7): 2348-57.
2. Steinman RM. Linking innate to adaptive immunity through dendritic cells. *Novartis Found Symp* 2006; **279**: 101-9; discussion 9-13, 216-9.
3. Cousins DJ, McDonald J, Lee TH. Therapeutic approaches for control of transcription factors in allergic disease. *J Allergy Clin Immunol* 2008; **121**(4): 803-9; quiz 10-1.
4. Ivanov, II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 2006; **126**(6): 1121-33.
5. Kebir H, Ifergan I, Alvarez JI, et al. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann Neurol* 2009; **66**(3): 390-402.
6. Korn T, Bettelli E, Gao W, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 2007; **448**(7152): 484-7.
7. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005; **6**(11): 1123-32.
8. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 2009; **30**(5): 646-55.
9. Wilson NJ, Boniface K, Chan JR, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007; **8**(9): 950-7.
10. Bullens DM, Decraene A, Seys S, Dupont LJ. IL-17A in human respiratory diseases: innate or adaptive immunity? Clinical implications. *Clin Dev Immunol* 2013; **2013**: 840315.
11. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology* 2010; **129**(3): 311-21.
12. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* 2014; **41**(4): 529-42.
13. Nurieva RI, Chung Y, Martinez GJ, et al. Bcl6 mediates the development of T follicular helper cells. *Science* 2009; **325**(5943): 1001-5.
14. Kaplan MH, Hufford MM, Olson MR. The development and in vivo function of T helper 9 cells. *Nat Rev Immunol* 2015; **15**(5): 295-307.
15. Tripathi SK, Lahesmaa R. Transcriptional and epigenetic regulation of T-helper lineage specification. *Immunological reviews* 2014; **261**(1): 62-83.
16. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol* 2015; **16**(1): 45-56.
17. McKenzie AN. Type-2 innate lymphoid cells in asthma and allergy. *Ann Am Thorac Soc* 2014; **11 Suppl 5**: S263-70.
18. Barlow JL, Peel S, Fox J, et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *J Allergy Clin Immunol* 2013; **132**(4): 933-41.

19. Maazi H, Patel N, Sankaranarayanan I, et al. ICOS:ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. *Immunity* 2015; **42**(3): 538-51.
20. Bernink JH, Germar K, Spits H. The role of ILC2 in pathology of type 2 inflammatory diseases. *Current opinion in immunology* 2014; **31**: 115-20.
21. Murphy KM, Stockinger B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat Immunol* 2010; **11**(8): 674-80.
22. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science*; **327**(5969): 1098-102.
23. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008; **8**(7): 523-32.
24. Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med* 1985; **161**(1): 72-87.
25. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 2001; **27**(1): 68-73.
26. Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked: forkhead box protein 3 mutations and lack of regulatory T cells. *J Allergy Clin Immunol* 2007; **120**(4): 744-50; quiz 51-2.
27. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008; **133**(5): 775-87.
28. Aschenbrenner K, D'Cruz LM, Vollmann EH, et al. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat Immunol* 2007; **8**(4): 351-8.
29. Spence PJ, Green EA. Foxp3+ regulatory T cells promiscuously accept thymic signals critical for their development. *Proc Natl Acad Sci U S A* 2008; **105**(3): 973-8.
30. Liston A, Nutsch KM, Farr AG, et al. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. *Proc Natl Acad Sci U S A* 2008; **105**(33): 11903-8.
31. Malek TR, Yu A, Vincek V, Scibelli P, Kong L. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 2002; **17**(2): 167-78.
32. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* 2007; **8**(12): 1353-62.
33. Banham AH, Powrie FM, Suri-Payer E. FOXP3+ regulatory T cells: Current controversies and future perspectives. *European journal of immunology* 2006; **36**(11): 2832-6.
34. Banham AH. Cell-surface IL-7 receptor expression facilitates the purification of FOXP3(+) regulatory T cells. *Trends Immunol* 2006; **27**(12): 541-4.
35. Akbari O, Freeman GJ, Meyer EH, et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* 2002; **8**(9): 1024-32.
36. Ostroukhova M, Seguin-Devaux C, Oriss TB, et al. Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest* 2004; **114**(1): 28-38.
37. Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. *Immunity* 2009; **31**(3): 438-49.

38. Curotto de Lafaille MA, Kutchukhidze N, Shen S, Ding Y, Yee H, Lafaille JJ. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* 2008; **29**(1): 114-26.
39. Urry Z, Xystrakis E, Richards DF, et al. Ligation of TLR9 induced on human IL-10-secreting Tregs by 1alpha,25-dihydroxyvitamin D3 abrogates regulatory function. *J Clin Invest* 2009; **119**(2): 387-98.
40. Paiva RS, Lino AC, Bergman ML, et al. Recent thymic emigrants are the preferential precursors of regulatory T cells differentiated in the periphery. *Proc Natl Acad Sci U S A* 2013; **110**(16): 6494-9.
41. Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood* 2005; **105**(3): 1162-9.
42. Barrat FJ, Cua DJ, Boonstra A, et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 2002; **195**(5): 603-16.
43. Barchet W, Price JD, Cella M, et al. Complement-induced regulatory T cells suppress T-cell responses but allow for dendritic-cell maturation. *Blood* 2006; **107**(4): 1497-504.
44. Hawrylowicz CM, O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* 2005; **5**(4): 271-83.
45. Tao R, de Zoeten EF, Ozkaynak E, et al. Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med* 2007; **13**(11): 1299-307.
46. Baron U, Floess S, Wieczorek G, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *European journal of immunology* 2007; **37**(9): 2378-89.
47. Lal G, Zhang N, van der Touw W, et al. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J Immunol* 2009; **182**(1): 259-73.
48. Akbar AN, Vukmanovic-Stejic M, Taams LS, Macallan DC. The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. *Nat Rev Immunol* 2007; **7**(3): 231-7.
49. Vukmanovic-Stejic M, Zhang Y, Cook JE, et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest* 2006; **116**(9): 2423-33.
50. Mantel PY, Kuipers H, Boyman O, et al. GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. *PLoS Biol* 2007; **5**(12): e329.
51. Wei J, Duramad O, Perng OA, Reiner SL, Liu YJ, Qin FX. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2007; **104**(46): 18169-74.
52. Zhou L, Lopes JE, Chong MM, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 2008; **453**(7192): 236-40.
53. Rubtsov YP, Rasmussen JP, Chi EY, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 2008; **28**(4): 546-58.
54. Holgate ST, Polosa R. Treatment strategies for allergy and asthma. *Nat Rev Immunol* 2008; **8**(3): 218-30.

55. Platts-Mills TA. The allergy epidemics: 1870-2010. *J Allergy Clin Immunol* 2015; **136**(1): 3-13.
56. . http://www.who.int/mediacentre/factsheets/fs307/en/ (accessed 2015 2015).
57. Containing the allergy epidemic: summary and recommendations of a new report from the Royal College of Physicians. *Clin Exp Allergy* 2004; **34**(4): 515-7.
58. Custovic A, Johnston SL, Pavord I, et al. EAACI position statement on asthma exacerbations and severe asthma. *Allergy* 2013; **68**(12): 1520-31.
59. Du Toit G, Roberts G, Sayre PH, et al. Randomized trial of peanut consumption in infants at risk for peanut allergy. *N Engl J Med* 2015; **372**(9): 803-13.
60. Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989; **299**(6710): 1259-60.
61. Law M, Morris JK, Wald N, Luczynska C, Burney P. Changes in atopy over a quarter of a century, based on cross sectional data at three time periods. *BMJ* 2005; **330**(7501): 1187-8.
62. Thomsen SF. Epidemiology and natural history of atopic diseases. *Eur Clin Respir J* 2015; **2**.
63. Kramer U, Schmitz R, Ring J, Behrendt H. What can reunification of East and West Germany tell us about the cause of the allergy epidemic? *Clin Exp Allergy* 2015; **45**(1): 94-107.
64. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002; **296**(5567): 490-4.
65. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002; **347**(12): 911-20.
66. Busse WW, Lemanske RF, Jr. Asthma. *N Engl J Med* 2001; **344**(5): 350-62.
67. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012; **18**(5): 673-83.
68. Hui CC, Murphy DM, Neighbour H, et al. T cell-mediated induction of thymic stromal lymphopoietin in differentiated human primary bronchial epithelial cells. *Clin Exp Allergy* 2014; **44**(7): 953-64.
69. Wang YH, Angkasekwinai P, Lu N, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J Exp Med* 2007; **204**(8): 1837-47.
70. Savenije OE, Mahachie John JM, Granell R, et al. Association of IL33-IL-1 receptor-like 1 (IL1RL1) pathway polymorphisms with wheezing phenotypes and asthma in childhood. *J Allergy Clin Immunol* 2014; **134**(1): 170-7.
71. Kay AB. Allergy and allergic diseases. First of two parts. *N Engl J Med* 2001; **344**(1): 30-7.
72. Fajt ML, Wenzel SE. Asthma phenotypes and the use of biologic medications in asthma and allergic disease: the next steps toward personalized care. *J Allergy Clin Immunol* 2015; **135**(2): 299-310; quiz 1.
73. Ortega HG, Liu MC, Pavord ID, et al. Mepolizumab treatment in patients with severe eosinophilic asthma. *N Engl J Med* 2014; **371**(13): 1198-207.
74. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012; **18**(5): 716-25.
75. Carlisle JM. Cortisone (compound E); summary of its clinical uses. *Br Med J* 1950; **2**(4679): 590-5.
76. Foulds WS, Greaves DP, Herx-Heimer H, Kingdom LG. Hydrocortisone in treatment of allergic conjunctivitis, allergic rhinitis, and bronchial asthma. *Lancet* 1955; **268**(6857): 234-5.

77. Holgate ST. A brief history of asthma and its mechanisms to modern concepts of disease pathogenesis. *Allergy Asthma Immunol Res* 2010; **2**(3): 165-71.
78. Boardman C, Chachi L, Gavrila A, et al. Mechanisms of glucocorticoid action and insensitivity in airways disease. *Pulm Pharmacol Ther* 2014; **29**(2): 129-43.
79. Barnes PJ. Glucocorticosteroids: current and future directions. *Br J Pharmacol* 2011; **163**(1): 29-43.
80. Barnes PJ. Targeting histone deacetylase 2 in chronic obstructive pulmonary disease treatment. *Expert Opin Ther Targets* 2005; **9**(6): 1111-21.
81. "International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma." Kian Fan Chung, Sally E. Wenzel, Jan L. Brozek, Andrew Bush, Mario Castro, Peter J. Sterk, Ian M. Adcock, Eric D. Bateman, Elisabeth H. Bel, Eugene R. Bleecker, Louis-Philippe Boulet, Christopher Brightling, Pascal Chanez, Sven-Erik Dahlen, Ratko Djukanovic, Urs Frey, Mina Gaga, Peter Gibson, Qutayba Hamid, Nizar N. Jajour, Thais Mauad, Ronald L. Sorkness and W. Gerald Teague. Eur Respir J 2014; 43: 343-373. *European Respiratory Journal* 2014; **43**(4): 1216-.
82. Ito K, Chung KF, Adcock IM. Update on glucocorticoid action and resistance. *J Allergy Clin Immunol* 2006; **117**(3): 522-43.
83. Wenzel S, Ford L, Pearlman D, et al. Dupilumab in persistent asthma with elevated eosinophil levels. *N Engl J Med* 2013; **368**(26): 2455-66.
84. Corren J, Lemanske RF, Hanania NA, et al. Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011; **365**(12): 1088-98.
85. Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet* 2007; **370**(9596): 1422-31.
86. Piper E, Brightling C, Niven R, et al. A phase II placebo-controlled study of tralokinumab in moderate-to-severe asthma. *Eur Respir J* 2013; **41**(2): 330-8.
87. Castro M, Zangrilli J, Wechsler ME, et al. Reslizumab for inadequately controlled asthma with elevated blood eosinophil counts: results from two multicentre, parallel, double-blind, randomised, placebo-controlled, phase 3 trials. *Lancet Respir Med* 2015; **3**(5): 355-66.
88. Laviolette M, Gossage DL, Gauvreau G, et al. Effects of benralizumab on airway eosinophils in asthmatic patients with sputum eosinophilia. *J Allergy Clin Immunol* 2013; **132**(5): 1086-96 e5.
89. Humbert M, Beasley R, Ayres J, et al. Benefits of omalizumab as add-on therapy in patients with severe persistent asthma who are inadequately controlled despite best available therapy (GINA 2002 step 4 treatment): INNOVATE. *Allergy* 2005; **60**(3): 309-16.
90. Boyman O, Kaegi C, Akdis M, et al. EAACI IG Biologicals task force paper on the use of biologic agents in allergic disorders. *Allergy* 2015; **70**(7): 727-54.
91. Busse WW, Holgate S, Kerwin E, et al. Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. *Am J Respir Crit Care Med* 2013; **188**(11): 1294-302.
92. Wenzel SE, Barnes PJ, Bleecker ER, et al. A randomized, double-blind, placebo-controlled study of tumor necrosis factor-alpha blockade in severe persistent asthma. *Am J Respir Crit Care Med* 2009; **179**(7): 549-58.
93. Gauvreau GM, O'Byrne PM, Boulet LP, et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. *N Engl J Med* 2014; **370**(22): 2102-10.

94. Hawrylowicz C, Richards D, Loke TK, Corrigan C, Lee T. A defect in corticosteroid-induced IL-10 production in T lymphocytes from corticosteroid-resistant asthmatic patients. *J Allergy Clin Immunol* 2002; **109**(2): 369-70.
95. O'Garra A, Barrat FJ, Castro AG, Vicari A, Hawrylowicz C. Strategies for use of IL-10 or its antagonists in human disease. *Immunological reviews* 2008; **223**: 114-31.
96. Ling EM, Smith T, Nguyen XD, et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004; **363**(9409): 608-15.
97. Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. *Clin Exp Allergy* 2004; **34**(9): 1364-72.
98. Hartl D, Koller B, Mehlhorn AT, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 2007; **119**(5): 1258-66.
99. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005; **202**(11): 1539-47.
100. Larsen JN, Broge L, Jacobi H. Allergy immunotherapy: the future of allergy treatment. *Drug Discov Today* 2015.
101. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. *World Allergy Organ J* 2015; **8**(1): 17.
102. Migueres M, Davila I, Frati F, et al. Types of sensitization to aeroallergens: definitions, prevalences and impact on the diagnosis and treatment of allergic respiratory disease. *Clin Transl Allergy* 2014; **4**: 16.
103. Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 2006; **6**(10): 761-71.
104. Jutel M, Akdis CA. Immunological mechanisms of allergen-specific immunotherapy. *Allergy* 2011; **66**(6): 725-32.
105. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; **102**(1): 98-106.
106. Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. *J Allergy Clin Immunol* 2004; **113**(6): 1025-34; quiz 35.
107. Holick MF. Vitamin D deficiency. *N Engl J Med* 2007; **357**(3): 266-81.
108. Bouillon R, Bischoff-Ferrari H, Willett W. Vitamin D and health: perspectives from mice and man. *J Bone Miner Res* 2008; **23**(7): 974-9.
109. Dimeloe S, Nanzer A, Ryanna K, Hawrylowicz C. Regulatory T cells, inflammation and the allergic response-The role of glucocorticoids and Vitamin D. *J Steroid Biochem Mol Biol* 2010; **120**(2-3): 86-95.
110. Holick MF, Chen TC, Lu Z, Sauter E. Vitamin D and skin physiology: a D-lightful story. *J Bone Miner Res* 2007; **22 Suppl 2**: V28-33.
111. Lips P, van Schoor NM, de Jongh RT. Diet, sun, and lifestyle as determinants of vitamin D status. *Ann N Y Acad Sci* 2014; **1317**: 92-8.
112. Mann EH, Chambers ES, Pfeffer PE, Hawrylowicz CM. Immunoregulatory mechanisms of vitamin D relevant to respiratory health and asthma. *Ann N Y Acad Sci* 2014; **1317**: 57-69.
113. Bischoff-Ferrari HA, Giovannucci E, Willett WC, Dietrich T, Dawson-Hughes B. Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. *Am J Clin Nutr* 2006; **84**(1): 18-28.

114. Correale J, Ysrraelit MC, Gaitan MI. Immunomodulatory effects of Vitamin D in multiple sclerosis. *Brain* 2009.
115. Black PN, Scragg R. Relationship between serum 25-hydroxyvitamin d and pulmonary function in the third national health and nutrition examination survey. *Chest* 2005; **128**(6): 3792-8.
116. Litonjua AA, Weiss ST. Is vitamin D deficiency to blame for the asthma epidemic? *J Allergy Clin Immunol* 2007; **120**(5): 1031-5.
117. Janssens W, Lehouck A, Carremans C, Bouillon R, Mathieu C, Decramer M. Vitamin D beyond bones in chronic obstructive pulmonary disease: time to act. *Am J Respir Crit Care Med* 2009; **179**(8): 630-6.
118. Chishimba L, Thickett DR, Stockley RA, Wood AM. The vitamin D axis in the lung: a key role for vitamin D-binding protein. *Thorax* 2010; **65**(5): 456-62.
119. Ginde AA, Mansbach JM, Camargo CA, Jr. Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. *Arch Intern Med* 2009; **169**(4): 384-90.
120. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol* 2001; **167**(9): 4974-80.
121. Wittke A, Weaver V, Mahon BD, August A, Cantorna MT. Vitamin D receptor-deficient mice fail to develop experimental allergic asthma. *J Immunol* 2004; **173**(5): 3432-6.
122. Gupta A, Sjoukes A, Richards D, et al. Relationship between serum vitamin D, disease severity, and airway remodeling in children with asthma. *Am J Respir Crit Care Med* 2011; **184**(12): 1342-9.
123. Sharief S, Jariwala S, Kumar J, Muntner P, Melamed ML. Vitamin D levels and food and environmental allergies in the United States: results from the National Health and Nutrition Examination Survey 2005-2006. *J Allergy Clin Immunol* 2011; **127**(5): 1195-202.
124. Calton EK, Keane KN, Newsholme P, Soares MJ. The Impact of Vitamin D Levels on Inflammatory Status: A Systematic Review of Immune Cell Studies. *PLoS One* 2015; **10**(11): e0141770.
125. Sadeghi K, Wessner B, Laggner U, et al. Vitamin D3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns. *European journal of immunology* 2006; **36**(2): 361-70.
126. Peric M, Koglin S, Kim SM, et al. IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. *J Immunol* 2008; **181**(12): 8504-12.
127. Dimitrov V, White JH. Species-specific regulation of innate immunity by vitamin D signaling. *J Steroid Biochem Mol Biol* 2015.
128. Barragan M, Good M, Kolls JK. Regulation of Dendritic Cell Function by Vitamin D. *Nutrients* 2015; **7**(9): 8127-51.
129. Lange NE, Litonjua A, Hawrylowicz CM, Weiss S. Vitamin D, the immune system and asthma. *Expert Rev Clin Immunol* 2009; **5**(6): 693-702.
130. Pfeffer PE, Chen YH, Woszczek G, et al. Vitamin D enhances production of soluble ST2, inhibiting the action of IL-33. *J Allergy Clin Immunol* 2015; **135**(3): 824-7 e3.
131. Xystrakis E, Kusumakar S, Boswell S, et al. Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. *J Clin Invest* 2006; **116**(1): 146-55.

132. Chambers ES, Hawrylowicz CM. The impact of vitamin D on regulatory T cells. *Curr Allergy Asthma Rep* 2011; **11**(1): 29-36.
133. Barnes PJ. Pathophysiology of allergic inflammation. *Immunological reviews* 2011; **242**(1): 31-50.
134. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997; **89**(4): 587-96.
135. Gregori S, Goudy KS, Roncarolo MG. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. *Front Immunol* 2012; **3**: 30.
136. Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004; **199**(11): 1567-75.
137. Hawrylowicz CM. Regulatory T cells and IL-10 in allergic inflammation. *J Exp Med* 2005; **202**(11): 1459-63.
138. Singer BD, King LS, D'Alessio FR. Regulatory T cells as immunotherapy. *Front Immunol* 2014; **5**: 46.
139. Saraiva M, Christensen JR, Veldhoen M, Murphy TL, Murphy KM, O'Garra A. Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. *Immunity* 2009; **31**(2): 209-19.
140. Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *J Exp Med* 2008; **205**(12): 2887-98.
141. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: Multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014; **133**(3): 621-31.
142. Vieira PL, Christensen JR, Minaee S, et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol* 2004; **172**(10): 5986-93.
143. Cousins DJ, Lee TH, Staynov DZ. Cytokine coexpression during human Th1/Th2 cell differentiation: direct evidence for coordinated expression of Th2 cytokines. *J Immunol* 2002; **169**(5): 2498-506.
144. Urry Z, Chambers ES, Xystrakis E, et al. The role of 1alpha,25-dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3+ and IL-10+ CD4+ T cells. *European journal of immunology* 2012; **42**(10): 2697-708.
145. Wang YH, Voo KS, Liu B, et al. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J Exp Med* 2010; **207**(11): 2479-91.
146. Cao S, Liu J, Song L, Ma X. The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. *J Immunol* 2005; **174**(6): 3484-92.
147. Apetoh L, Quintana FJ, Pot C, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol* 2010; **11**(9): 854-61.
148. Mutalithas K, Guillen C, Day C, Brightling CE, Pavord ID, Wardlaw AJ. CRTH2 expression on T cells in asthma. *Clinical and experimental immunology* 2010; **161**(1): 34-40.
149. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**(5609): 1057-61.

150. Silverman GA, Bird PI, Carrell RW, et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *The Journal of biological chemistry* 2001; **276**(36): 33293-6.
151. Midwood KS, Schwarzbauer JE. Tenascin-C modulates matrix contraction via focal adhesion kinase- and Rho-mediated signaling pathways. *Molecular biology of the cell* 2002; **13**(10): 3601-13.
152. Mocchegiani E, Giacconi R, Costarelli L. Metalloproteases/anti-metalloproteases imbalance in chronic obstructive pulmonary disease: genetic factors and treatment implications. *Current opinion in pulmonary medicine* 2011; **17 Suppl 1**: S11-9.
153. Reid DM, Gow NA, Brown GD. Pattern recognition: recent insights from Dectin-1. *Current opinion in immunology* 2009; **21**(1): 30-7.
154. Lombardi V, Van Overtvelt L, Horiot S, et al. Toll-like receptor 2 agonist Pam3CSK4 enhances the induction of antigen-specific tolerance via the sublingual route. *Clin Exp Allergy* 2008; **38**(11): 1819-29.
155. Kawaguchi M, Fujita J, Kokubu F, et al. Induction of insulin-like growth factor-I by interleukin-17F in bronchial epithelial cells. *Clin Exp Allergy* 2010; **40**(7): 1036-43.
156. Ashton-Rickardt PG. An emerging role for Serine Protease Inhibitors in T lymphocyte immunity and beyond. *Immunology letters* 2013; **152**(1): 65-76.
157. Janciauskiene SM, Nita IM, Stevens T. Alpha1-antitrypsin, old dog, new tricks. Alpha1-antitrypsin exerts in vitro anti-inflammatory activity in human monocytes by elevating cAMP. *The Journal of biological chemistry* 2007; **282**(12): 8573-82.
158. Yamane T, Takeuchi K, Yamamoto Y, et al. Legumain from bovine kidney: its purification, molecular cloning, immunohistochemical localization and degradation of annexin II and vitamin D-binding protein. *Biochim Biophys Acta* 2002; **1596**(1): 108-20.
159. Probst-Kepper M, Geffers R, Kroger A, et al. GARP: a key receptor controlling FOXP3 in human regulatory T cells. *J Cell Mol Med* 2009; **13**(9B): 3343-57.
160. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* 2004; **104**(9): 2840-8.
161. Loebbermann J, Thornton H, Durant L, et al. Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection. *Mucosal Immunol* 2012; **5**(2): 161-72.
162. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *European journal of immunology* 2009; **39**(11): 3147-59.
163. Sigmundsdottir H, Pan J, Debes GF, et al. DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. *Nat Immunol* 2007; **8**(3): 285-93.
164. Sriram U, Xu J, Chain RW, et al. IL-4 Suppresses the Responses to TLR7 and TLR9 Stimulation and Increases the Permissiveness to Retroviral Infection of Murine Conventional Dendritic Cells. *PLoS One* 2014; **9**(1): e87668.
165. Mueller T, Terada T, Rosenberg IM, Shibolet O, Podolsky DK. Th2 cytokines down-regulate TLR expression and function in human intestinal epithelial cells. *J Immunol* 2006; **176**(10): 5805-14.

166. Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* 2003; **4**(7): 670-9.
167. Matsumoto K, Inoue H, Nakano T, et al. B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism. *J Immunol* 2004; **172**(4): 2530-41.
168. Xiao Y, Yu S, Zhu B, et al. RGMb is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance. *J Exp Med* 2014; **211**(5): 943-59.
169. Tree TI, Lawson J, Edwards H, et al. Naturally arising human CD4 T-cells that recognize islet autoantigens and secrete interleukin-10 regulate proinflammatory T-cell responses via linked suppression. *Diabetes* 2010; **59**(6): 1451-60.
170. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *Journal of immunology* 2005; **174**(4): 1783-6.
171. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol* 2015; **15**(6): 388-400.
172. Willoughby CA, Bull HG, Garcia-Calvo M, Jiang J, Chapman KT, Thornberry NA. Discovery of potent, selective human granzyme B inhibitors that inhibit CTL mediated apoptosis. *Bioorg Med Chem Lett* 2002; **12**(16): 2197-200.
173. Vrazo AC, Hontz AE, Figueira SK, et al. Live cell evaluation of granzyme delivery and death receptor signaling in tumor cells targeted by human natural killer cells. *Blood* 2015; **126**(8): e1-e10.
174. Gehrmann M, Stangl S, Kirschner A, et al. Immunotherapeutic targeting of membrane Hsp70-expressing tumors using recombinant human granzyme B. *PLoS One* 2012; **7**(7): e41341.
175. Fellows E, Gil-Parrado S, Jenne DE, Kurschus FC. Natural killer cell-derived human granzyme H induces an alternative, caspase-independent cell-death program. *Blood* 2007; **110**(2): 544-52.
176. Ewen CL, Kane KP, Bleackley RC. Granzyme H induces cell death primarily via a Bcl-2-sensitive mitochondrial cell death pathway that does not require direct Bid activation. *Mol Immunol* 2013; **54**(3-4): 309-18.
177. Chung KF, Wenzel SE, Brozek JL, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014; **43**(2): 343-73.
178. Vazquez-Tello A, Semlali A, Chakir J, et al. Induction of glucocorticoid receptor-beta expression in epithelial cells of asthmatic airways by T-helper type 17 cytokines. *Clin Exp Allergy* 2010; **40**(9): 1312-22.
179. Acosta-Rodriguez EV, Rivino L, Geginat J, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 2007; **8**(6): 639-46.
180. Eyerich S, Eyerich K, Pennino D, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest* 2009; **119**(12): 3573-85.
181. Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 2003; **97**(6): 726-33.
182. Molet S, Hamid Q, Davoine F, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001; **108**(3): 430-8.
183. Al-Ramli W, Prefontaine D, Chouiali F, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* 2009; **123**(5): 1185-7.

184. Chakir J, Shannon J, Molet S, et al. Airway remodeling-associated mediators in moderate to severe asthma: Effect of steroids on TGF-β, IL-11, IL-17, and type I and type III collagen expression. *Journal of Allergy and Clinical Immunology* 2003; **111**(6): 1293-8.
185. Bullens DM, Truyen E, Coteur L, et al. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res* 2006; **7**: 135.
186. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir Med* 2010; **104**(8): 1131-7.
187. Lloyd CM, Saglani S. T cells in asthma: influences of genetics, environment, and T-cell plasticity. *J Allergy Clin Immunol* 2013; **131**(5): 1267-74; quiz 75.
188. Roussel L, Houle F, Chan C, et al. IL-17 promotes p38 MAPK-dependent endothelial activation enhancing neutrophil recruitment to sites of inflammation. *J Immunol* 2010; **184**(8): 4531-7.
189. Bellini A, Marini MA, Bianchetti L, Barczyk M, Schmidt M, Mattoli S. Interleukin (IL)-4, IL-13, and IL-17A differentially affect the profibrotic and proinflammatory functions of fibrocytes from asthmatic patients. *Mucosal Immunol* 2012; **5**(2): 140-9.
190. Zielinski CE, Mele F, Aschenbrenner D, et al. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature* 2012; **484**(7395): 514-8.
191. Essilfie AT, Simpson JL, Horvat JC, et al. Haemophilus influenzae infection drives IL-17-mediated neutrophilic allergic airways disease. *PLoS Pathog* 2011; **7**(10): e1002244.
192. Essilfie AT, Horvat JC, Kim RY, et al. Macrolide therapy suppresses key features of experimental steroid-sensitive and steroid-insensitive asthma. *Thorax* 2015; **70**(5): 458-67.
193. Jirapongsananuruk O, Melamed I, Leung DY. Additive immunosuppressive effects of 1,25-dihydroxyvitamin D3 and corticosteroids on TH1, but not TH2, responses. *J Allergy Clin Immunol* 2000; **106**(5): 981-5.
194. Reynolds JM, Pappu BP, Peng J, et al. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity* 2010; **32**(5): 692-702.
195. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007; **8**(9): 942-9.
196. Boniface K, Blumenschein WM, Brovont-Porth K, et al. Human Th17 cells comprise heterogeneous subsets including IFN-gamma-producing cells with distinct properties from the Th1 lineage. *J Immunol* 2010; **185**(1): 679-87.
197. Annunziato F, Cosmi L, Santarlasci V, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007; **204**(8): 1849-61.
198. Sommer A, Fabri M. Vitamin D Regulates Cytokine Patterns Secreted by Dendritic Cells to Promote Differentiation of IL-22-Producing T Cells. *PLoS One* 2015; **10**(6): e0130395.
199. Nanzer AM, Chambers ES, Ryanna K, et al. Enhanced production of IL-17A in patients with severe asthma is inhibited by 1alpha,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. *J Allergy Clin Immunol* 2013; **132**(2): 297-304 e3.

200. Taher YA, van Esch BC, Hofman GA, Henricks PA, van Oosterhout AJ. 1alpha,25-dihydroxyvitamin D3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF-beta. *J Immunol* 2008; **180**(8): 5211-21.
201. Heine G, Tabeling C, Hartmann B, et al. 25-hydroxvitamin D3 promotes the long-term effect of specific immunotherapy in a murine allergy model. *J Immunol* 2014; **193**(3): 1017-23.
202. Grundstrom J, Neimert-Andersson T, Kemi C, et al. Covalent coupling of vitamin D3 to the major cat allergen Fel d 1 improves the effects of allergen-specific immunotherapy in a mouse model for cat allergy. *Int Arch Allergy Immunol* 2012; **157**(2): 136-46.
203. Majak P, Rychlik B, Stelmach I. The effect of oral steroids with and without vitamin D3 on early efficacy of immunotherapy in asthmatic children. *Clin Exp Allergy* 2009; **39**(12): 1830-41.
204. Baris S, Kiykim A, Ozen A, Tulunay A, Karakoc-Aydiner E, Barlan IB. Vitamin D as an adjunct to subcutaneous allergen immunotherapy in asthmatic children sensitized to house dust mite. *Allergy* 2014; **69**(2): 246-53.
205. Chambers ES, Nanzer AM, Pfeffer PE, et al. Distinct endotypes of steroid-resistant asthma characterized by IL-17A and IFN-gamma immunophenotypes: Potential benefits of calcitriol. *J Allergy Clin Immunol* 2015.
206. Van Hemelen D, Mahler V, Fischer G, et al. HLA class II peptide tetramers vs allergen-induced proliferation for identification of allergen-specific CD4 T cells. *Allergy* 2015; **70**(1): 49-58.
207. Wambre E, DeLong JH, James EA, et al. Specific immunotherapy modifies allergen-specific CD4(+) T-cell responses in an epitope-dependent manner. *J Allergy Clin Immunol* 2014; **133**(3): 872-9 e7.
208. Komatsu N, Okamoto K, Sawa S, et al. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat Med* 2014; **20**(1): 62-8.
209. Wensink AC, Hack CE, Bovenschen N. Granzymes regulate proinflammatory cytokine responses. *J Immunol* 2015; **194**(2): 491-7.

Publications by K Ryanna arising from or related to this work

Nanzer AM, Chambers ES, Ryanna K, et al. Enhanced production of IL-17A in patients with severe asthma is inhibited by 1alpha,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. *J Allergy Clin Immunol* 2013; **132**(2): 297-304 e3.

Ryanna K, Stratigou V, Safinia N, Hawrylowicz C. Regulatory T cells in bronchial asthma. *Allergy* 2009; **64**(3): 335-47.

Gupta A, Dimeloe S, Richards DF, et al. Defective IL-10 expression and in vitro steroid-induced IL-17A in paediatric severe therapy-resistant asthma. *Thorax* 2014; **69**(6): 508-15.

Chambers ES, Nanzer AM, Richards DF, et al. Serum 25-dihydroxyvitamin D levels correlate with CD4(+)Foxp3(+) T-cell numbers in moderate/severe asthma. *J Allergy Clin Immunol* 2012; **130**(2): 542-4.

Nanzer AM, Chambers ES, Ryanna K, et al. The effects of calcitriol treatment in glucocorticoid-resistant asthma. *J Allergy Clin Immunol* 2014; **133**(6): 1755-7 e4.

Dimeloe S, Nanzer A, Ryanna K, Hawrylowicz C. Regulatory T cells, inflammation and the allergic response-The role of glucocorticoids and Vitamin D.
J Steroid Biochem Mol Biol. 2010 120(2-3):86-95.

Hawrylowicz C, Ryanna K. Asthma and allergy: the early beginnings.
Nat Med. 2010 16(3):274-5.